EFFECTS OF LONG-TERM HORMONE TREATMENT ON COGNITIVE BEHAVIOR AND THE STRUCTURE OF THE MEDIAL PREFRONTAL CORTEX DURING AGING IN FEMALE RATS

BY

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DISSERTATION

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ABSTRACT

Although previous research has indicated that hormone replacement therapy benefits memory in menopausal women, several newer studies have shown no effect or detrimental effects. These inconsistencies emphasize the need to evaluate the role of hormones in protecting against age-related cognitive decline in an animal model. Furthermore, research has found that ovarian hormones alter brain structure and function. However, many studies evaluating the effects of estrogen and progesterone on brain structure have used young adult animals and have not administered medroxyprogesterone acetate (MPA), the most commonly prescribed progestin. The aging brain may respond differently to the presence of these hormones. Therefore, the effects of long-term hormone treatment during aging on cognition and neuroanatomy were investigated. Female Long Evans hooded rats were ovariectomized at middle age (12-14 months) and placed in one of 5 groups: no replacement, chronic estrogen only, chronic estrogen and progesterone, chronic estrogen and MPA, and cyclic estrogen. Hormone treatment continued until sacrifice. Estrogen was administered in the drinking water. Progesterone and MPA were administered with subcutaneous pellets. Following five months of hormone replacement, animals were tested on a delayed alternation task in the T-maze. Two weeks after completing the T-maze animals were tested in the Morris water maze. At approximately 20 months of age, animals were sacrificed and their brains were sectioned and using immunohistochemistry, stained for tyrosine hydroxylase and synaptophysin. Adjacent sections were Nissl stained to calculate volume and quantify neuron number. The medial prefrontal cortex was examined because it is involved in several cognitive tasks and is known to be sensitive to both aging and ovarian hormones. Using unbiased stereology and light microscopy, neuron number and synaptophysin labeled boutons

were quantified. Images were acquired of the tyrosine hydroxylase sections using Axiovision (Zeiss) on a fluorescent microscope and fiber densities were quantified.

Behavioral results found that animals receiving estrogen in combination with MPA acquired the t-maze faster than no replacement animals, but there were no differences in performance on the delayed portion of the task. However, on the Morris water maze, animals receiving this hormone treatment were impaired as compared to other hormone treated groups. Estrogen in combination with MPA resulted in greater synaptophysin levels in the medial prefrontal cortex. Analysis of tyrosine hydroxylase fibers found that animals receiving estrogen in combination with MPA consistently had significantly higher tyrosine hydroxylase pixel densities than no replacement animals. Hormone treatment did not significantly alter neuron number in the medial prefrontal cortex.

These results indicate that the effects of long-term hormone treatment are task specific and long-term hormone treatments alter dopaminergic functioning and synapse number in the medial prefrontal cortex, providing a possible mechanism by which long-term hormone treatments can influence cognition. Importantly, these beneficial neural outcomes were observed in groups receiving estrogen in combination with the controversial progestin, MPA.

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PREFACE

Menopause in humans is associated with a loss of ovarian hormones and this decline in estrogen and progesterone has been linked to several of the symptoms related to menopause. Hormone therapies including Premarin (conjugated equine estrogens; CEE) and Prempro (CEE in combination with medroxyprogesterone acetate; MPA), have been approved to alleviate these symptoms. In women with a uterus, MPA, a synthetic analogue of progesterone, is administered in combination with estrogen therapy to prevent endometrial hyperplasia (Whitehead, King, McQueen, & Campbell, 1979). Along with alleviating some of the symptoms of menopause, studies have found beneficial effects of hormone treatment on cognition (Joffe et al., 2006; Krug, Born, & Rasch, 2006; LeBlanc, Janowsky, Chan, & Nelson, 2001). However, results from the Women's Health Initiative, indicate that CEE alone or CEE administered with MPA results in an increased risk of stroke, and dementia (Anderson et al., 2004; Anderson et al., 2004; Shumaker et al., 2004; Wassertheil-Smoller et al., 2003). Whether hormone treatment alters brain structure or has beneficial effects on cognition during aging has recently become a topic of debate. Indentifying the underlying mechanisms that contribute to cognitive changes occurring during aging provides potential targets for treatments aimed at protecting against this age-related decline. However, there are many factors that may affect the neural and behavioral outcomes of hormone replacement during aging, including, whether or not estrogen is combined with progesterone and if so the type of progesterone used, natural or synthetic, the mode of administration, length of hormone deprivation, and route of administration.

Studies have found that different combinations of hormone treatment do not always result in similar outcomes. The addition of progestogens can result in different results from estrogen only treatments. Progesterone completely reversed the beneficial effects of estrogen on the water

maze so that middle-aged animals receiving both estrogen and progesterone did not differ from animals not receiving hormone replacement (Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006). Also, the type of progestogen used can influence the outcome and many animal studies that have evaluated the effects of progestogens on cognition have used progesterone rather than MPA, the most commonly prescribed progestin. While MPA is a synthetic analogue of progesterone and is an agonist at progesterone receptors, it also binds to androgen and glucocorticoid receptors (Bamberger, Else, Bamberger, Beil, & Schulte, 1999; Bardin, Brown, Isomaa, & Janne, 1983). Some argue that natural progesterone may be more beneficial than MPA. One study found that progesterone was neuroprotective in vivo while the synthetic progestin MPA was not (Ciriza, Carrero, Frye, & Garcia-Segura, 2006), indicating that the type of progesterone used could determine whether the result of hormone replacement is beneficial. A recent study found that MPA administered without estrogen impaired performance on the water radial arm maze and the water maze (Braden et al., 2010).

Another factor that may influence the outcomes observed with hormone treatment is the mode of replacement. It is possible that cyclic hormone replacement may be more beneficial than chronic hormone replacement because it more closely resembles the natural cycle of hormones. The studies that have investigated the effects of repeated cyclic hormone treatment are inconsistent. Weekly injections of estradiol plus progesterone enhanced performance of aged female rats on a delayed match-to-position T-maze task (Gibbs, 2000) and in aged rhesus monkeys, cyclic estradiol cypionate injections improved spatial working memory performance (P. R. Rapp, Morrison, & Roberts, 2003). Two other studies have found that both continuous and intermittent estradiol significantly improved task acquisition in middle aged and aged animals (Bimonte-Nelson et al., 2006; Markowska & Savonenko, 2002). However, cyclic administration of

estradiol benzoate did not improve performance in middle-aged female rats on a 12-arm radial arm maze (Ziegler & Gallagher, 2005). Furthermore, while continuous estradiol treatment had no effect on spatial working or reference memory in the radial arm maze, intermittent estradiol treatment impaired spatial reference memory (Gresack & Frick, 2006). Although the results of these studies differ, most of the studies that have attempted to mimic the natural cycle of hormones have only simulated certain aspects of the cycle. It is possible that more closely simulating the natural cycle by including fluctuations in both estrogen and progesterone levels may be more beneficial than the chronic treatment of ovarian hormones.

Recently the length of hormone deprivation has been extensively studied. This research has indicated that there is a "window of opportunity" meaning that waiting too long after menopause or estropause in rats, to begin hormone replacement could lead to the replacement having no effect on cognition. In the Women's Health Initiative previously discussed, the ages of the subjects ranged from 65-79 which may be too late to begin hormone replacement in order to have beneficial effects on cognition. There is evidence that the timing of hormone replacement onset is an important factor and may explain the negative findings of the Women's Health Initiative studies (Gibbs, 2000; Sherwin, 2009). In a study by Gibbs (2000), animals that had hormone replacement initiated immediately following ovariectomy or within three months of ovariectomy performed significantly better than controls, while animals that received hormone replacement 10 months after ovariectomy showed no significant differences from controls. Recent work indicates that the ability of estrogen to enhance synaptic communication in the hippocampus depends on the length of hormone deprivation not the age of the animal (McLaughlin, Bimonte-Nelson, Neisewander, & Conrad, 2008; C. C. Smith, Vedder, Nelson, Bredemann, & McMahon, 2010).

Oral administration is the most common route of administration of hormone treatment in post menopausal women. Animal studies more commonly use injections or silastic capsules to deliver hormone treatments. These routes of administration have different pharmacokinetics than oral administration and do not allow for first pass metabolism of the hormone which may result in different outcomes of hormone treatment. Indeed, the only study that has administered estradiol orally and examined effects on cognition in an animal model found improved performance on object recognition (Fernandez & Frick, 2004), while another study from the same lab found that daily injections of estradiol did not improve performance on the same task (Gresack & Frick, 2006). Therefore it is important to evaluate the effects of hormone treatment using a route of administration that is similar to the most common route used by post-menopausal women. Although studies have attempted to address some of these factors, few have looked at the effects on hormone replacement in conjunction with one another. Several questions remain regarding the effects of hormone treatment on the structure and function of the brain during aging.

INTRODUCTION

In humans, aging is almost always accompanied by a decline in cognitive abilities. For women this decline has been associated with menopause. Several studies have found that women who receive estrogen replacement show improvement on a wide variety of cognitive tasks including tests of memory (reviewed by LeBlanc et al., 2001). However, others have found little or no cognitive benefit of when women were given estrogen only or estrogen plus medroxyprogesterone (Hogervorst, Williams, Budge, Riedel, & Jolles, 2000; Yaffe, Sawaya, Lieberburg, & Grady, 1998). Although the hippocampus has long been presumed the primary site of action of estrogens on cognition, research indicates that the prefrontal cortex (PFC) may be important as well (Joffe et al., 2006; Kampen & Sherwin, 1994; Keenan, Ezzat, Ginsburg, & Moore, 2001; Krug et al., 2006).

Like humans, rodents experience age-related cognitive decline, and thus provide models for testing the role ovarian hormones may play in cognition during the aging process. Estradiol or estradiol benzoate treatment in both middle aged and aged female rodents often improves performance on the water maze (Frick, Fernandez, & Bulinski, 2002; Frye, Rhodes, & Dudek, 2005; Harburger, Bennett, & Frick, 2007; Markham, Pych, & Juraska, 2002) however see (Foster, Sharrow, Kumar, & Masse, 2003). Although our lab previously found that chronic treatment with ovarian hormones improves performance on the water maze, a hippocampal dependant task, (Markham et al., 2002), prior to the study presented in chapter 1, it was not known how estrogen in combination with MPA would affect performance on this task. Also, this was the first study to examine the effects of long-term chronic hormone treatment on the water maze.

Studies have also found improved performance after estrogen treatment on several other tasks including the radial arm maze, spontaneous alternation, and both delayed matching to position and delayed non-matching to position tasks (Gibbs, 2000; Heikkinen, Puolivali, & Tanila, 2004; Markowska & Savonenko, 2002; M. M. Miller et al., 1999). However, some studies have found that the behavioral outcome of hormone treatments depends on the type of task used (Frye & Walf, 2008). Whether the hormone combinations used in Chapter 1 would result in a similar outcome on delayed alternation, a task mediated by the PFC, was evaluated in Chapter 2. Research indicates that the PFC may be an important site for estrogen's actions on cognition (Keenan et al., 2001) and this brain region seems to be particularly vulnerable to aging. For example, brain metabolism is decreased during aging and the medial PFC experiences the largest decline (Pardo et al., 2007) and this brain region has greater decline in gray matter volume than other brain areas (Raz et al., 2005; Resnick, Pham, Kraut, Zonderman, & Davatzikos, 2003). Studies have found several cellular changes that could accompany this change in volume including, cell loss, reductions in dendritic material, and alterations to neurotransmitter systems (Cupp & Uemura, 1980; de Brabander, Kramers, & Uylings, 1998; Duan et al., 2003; Grill & Riddle, 2002; Inoue et al., 2001; Jacobs, Driscoll, & Schall, 1997; Kaasinen et al., 2000; Kaasinen et al., 2002; Markham & Juraska, 2002; Mizoguchi, Shoji, Tanaka, Maruyama, & Tabira, 2009; Wallace, Frankfurt, Arellanos, Inagaki, & Luine, 2007). Importantly, there have been few studies evaluating the effects of long-term chronic hormone treatment on the structure of the mPFC during aging. MRI studies in humans have found that hormone treatment decreases the shrinkage associated with aging of both the cortex and hippocampus (Boccardi et al., 2006; Lord, Engert, Lupien, & Pruessner, 2010; Resnick et al., 2009; Robertson et al., 2009). It is not possible to observe the structural changes that may be occurring at the cellular level in the human

MRI studies; however, in young non-human primates, estrogen treatment increases spine synapse density in the PFC (Hao et al., 2006; Leranth, Hajszan, Szigeti-Buck, Bober, & MacLusky, 2008; Tang et al., 2004), indicating that estradiol may affect the number of synapses in the aged PFC. In rodents, ovariectomy decreases spine density in the rat mPFC (Wallace, Luine, Arellanos, & Frankfurt, 2006) and intact females lose fewer spines during aging in the medial prefrontal cortex (mPFC) than males which may be due to the continued secretion of estrogen and progesterone during estropause (Markham & Juraska, 2002). Studies indicate that estrogen alters spines in the hippocampus of young animals, but it is unknown if estrogen alters synapses in the aging rat mPFC. In addition, comparison of the effects of different progestogens has not been conducted. Chapter 3 examines the effects of different hormone formulations during aging on synapses in the mPFC.

Changes in several neurotransmitter systems could underlie the effects of hormone treatment on synapse number. However, the dopaminergic system is important for prefrontal functioning and altered with aging. Furthermore, studies suggest that ovarian hormones also affect dopaminergic function. Low dose estrogen treatment increased dopamine levels in postmenopausal women (Zarate, Fonseca, Ochoa, Basurto, & Hernandez, 2002) and in non-human primates, ovariectomy reduced, and estrogen replacement restored the density of axons stained for tyrosine hydroxylase in the dorsolateral PFC (Kritzer & Kohama, 1998). In rats, estrogen treatment influences the dopaminergic system (Inagaki, Gautreaux, & Luine, 2010; V. N. Luine, Richards, Wu, & Beck, 1998; McDermott, 1993). However, it is currently unknown how long-term hormone treatment alters dopaminergic functioning in the mPFC. Chapter 4 examines the question of whether hormone treatment during aging alters the density of dopaminergic fibers in the mPFC.

An alternative mechanism by which hormone treatment decreases shrinkage associated with aging is by preventing neuron loss. Studies have found a loss of cortical neurons in humans (Pakkenberg & Gundersen, 1997), rhesus monkeys (D. E. Smith, Rapp, McKay, Roberts, & Tuszynski, 2004), and rats (Yates, Markham, Anderson, Morris, & Juraska, 2008) during aging. There is evidence from our lab that this loss is sexually dimorphic with males but not females losing neurons during aging in the mPFC (Yates et al., 2008). The presence of ovarian hormones may protect females from this age-related loss. In addition, estrogen and progesterone have been shown to have neuroprotective properties in vitro; however, MPA counteracts the neuroprotective effects of estrogen (Nilsen & Brinton, 2002b). Currently, it is unknown what this might mean for neuron number during aging. Chapter 4 also examined neuron number in the mPFC after long-term chronic exposure to these hormones.

CHAPTER 1

LONG-TERM REPLACEMENT OF ESTROGEN WITH MEDROXYPROGESTERONE IMPAIRS PERFORMANCE ON THE MORRIS WATER MAZE IN MIDDLE AGED FEMALE RATS [1]

[1] Previously published as: Lowry NC, Pardon LP, Yates MA, Juraska JM (2010) Effects of long-term treatment with 17 beta-estradiol and medroxyprogesterone acetate on water maze performance in middle aged female rats. Horm Behav 58:200-207

Abstract

Although previous research has indicated that hormone replacement therapy benefits memory in menopausal women, several recent studies have shown either detrimental or no effects of treatment. These inconsistencies emphasize the need to evaluate the role of ovarian hormones in protecting against age-related cognitive decline in an animal model. The present study investigated the effects of long-term hormone treatment during aging on the Morris water maze. Female Long Evans hooded rats were ovariectomized at middle age (12-13 months) and were immediately placed in one of five groups: no replacement, chronic 17 β-estradiol (E_2) only, chronic E_2 and progesterone, chronic E_2 and medroxyprogesterone acetate (MPA), or cyclic E_2 only. E_2 was administered in the drinking water in either a chronic or cyclic (3 out of 4 days) fashion. Progesterone and MPA were administered via subcutaneous pellets. Following 6 months of hormone treatment, animals were tested on the Morris water maze. Animals performed four trials a day for 4 days and after the final day of testing a subset of animals completed a probe trial. Across 4 days of testing, rats receiving E_2 in combination with MPA performed significantly worse than all other groups receiving hormone replacement. In addition on the last day of testing, chronic E_2 administration was more beneficial than cyclic administration and no replacement. Thus, compared to other hormone-treated groups, long-term treatment with E_2 in combination with MPA resulted in impaired performance on the spatial Morris water maze.

Introduction

A decline in many cognitive abilities accompanies aging in humans. For women, this decline has been associated with the decrease in ovarian hormones that occurs during menopause (Nappi et al., 1999; Sherwin, 1988). Numerous studies have reported an improvement in cognitive performance of women receiving hormone treatment (reviewed by LeBlanc et al., 2001). For example, hormone treatment enhances measures of verbal memory and improves performance on tasks of spatial and verbal working memory (Carlson & Sherwin, 1998; Duff & Hampson, 2000; Kampen & Sherwin, 1994). However, other studies have found little or no cognitive benefit of hormone treatment in postmenopausal women (reviewed by Hogervorst et al., 2000). The Women's Health Initiative found that conjugated equine estrogen alone or administered with medroxyprogesterone acetate (MPA), the most common progestin given to women, failed to enhance cognition and increased the number of subjects diagnosed with either probable dementia or mild cognitive impairment in post menopausal women (Espeland et al., 2004; S. R. Rapp et al., 2003; Shumaker et al., 2003; Shumaker et al., 2004). These conflicting results emphasize the need to determine the factors influencing whether hormone replacement benefits or impairs cognition.

Studies investigating the association between hormone treatment and cognition in humans contain confounds which can be manipulated or controlled in a rodent model. Rodents experience age-related cognitive decline and thus may provide useful models for explaining the role ovarian hormones play in cognition during the aging process. However, most research investigating the effects of hormone treatment on cognition has used young animals (reviewed in Daniel, 2006; J. M. Juraska & Rubinow, 2008), which does not reflect the possible interaction of hormone treatment with aging. Indeed, studies suggest that the effects of hormone treatment in

young female animals are often not the same as the effects of hormone treatment during aging. Our laboratory found that young adult females who were ovariectomized and given 17 β-estradiol (E_2) and progesterone were impaired in the acquisition of the Morris water maze (Chesler & Juraska, 2000), while replacement of E_2 and progesterone in middle aged animals facilitated performance of the same task (Markham et al., 2002). Other laboratories have also found hormone by age interactions in females within the same study (Foster et al., 2003; Talboom, Williams, Baxley, West, & Bimonte-Nelson, 2008). Therefore, it is important to assess the effects of hormone treatment in middle aged or aged female animals, a model that more closely mimics hormone treatment during human menopause. Recently, studies have used middle aged or aged female animals when evaluating the cognitive effects of various types of hormone treatment. These studies have found improved performance after E_2 treatment on the radial arm maze, spontaneous alternation, and both delayed matching to position and delayed non-matching to position tasks (Gibbs, 2000; Heikkinen et al., 2004; Markowska & Savonenko, 2002; M. M. Miller et al., 1999). E_2 or estradiol benzoate treatment in both middle aged and aged female rodents often improves performance on the Morris water maze (Frick et al., 2002; Frye et al., 2005; Harburger et al., 2007; Markham et al., 2002). However, Foster et al., (2003) found no effect of estradiol benzoate on acquisition of the water maze in aged animals. Also, 5 weeks of oral E_2 administration in middle aged animals resulted in impairment on the radial arm maze (Fernandez & Frick, 2004). In addition, in aged animals, chronic E_2 had no effect on working memory errors in the radial arm maze (Gresack & Frick, 2006). The inconsistencies in the literature could be due to several factors including the mode of administration (chronic or cyclic replacement), pharmacokinetics of the route of administration, whether or not estrogens are

combined with a progestogen, and the type of task that is used to assess the influence of hormone treatment on cognition.

The present study was designed to evaluate the effects of different modes of hormone treatment (chronic and cyclic) and different regimens of hormone treatment on a reference memory task that is hippocampal-dependent, the spatial Morris water maze. Middle aged female rats were ovariectomized, immediately given hormone treatment for 6 months, and subsequently tested on the Morris water maze. Because much of the animal research to this point has used E_2 rather than conjugated equine estrogen, we used E_2 in order to more directly compare our results. Four types of treatment were administered: chronic E_2 alone, chronic E_2 and progesterone, chronic E_2 and MPA and cyclic E_2 alone. Directly comparing the groups with progesterone to those with MPA should help determine if these progestogens differ in preventing the cognitive decline associated with aging. Comparing these hormone treatments with E_2 only treatments will indicate if the addition of a progestogen impacts the outcome of hormone treatments on age related cognitive decline. We predicted that middle aged females receiving hormone treatment would perform better than animals not receiving hormones. Furthermore, we hypothesized that cyclic E_2 treatment, which is closer to the natural cycle, would be more beneficial for this task than chronic E_2 treatment.

Methods

Subjects

With the exception of the number of subjects, these procedures were used for all chapters regarding hormone treatment. Subjects were 47 female Long Evans hooded rats purchased from Charles River Laboratories as retired breeders at the age of 10–12 months. Due to the large number of subjects and groups, animals were run in two experimental cohorts. Animals from the

same group were pair- or, if necessary, triple housed, in clear Plexiglass cages in a temperature-controlled environment on a 12:12-hr light–dark cycle. Food and water were available *ad libitum* to all animals, except during the delayed T-maze task discussed in Chapter 2 during which the animals were maintained at 85–90% of their normal body weight. Prior to being tested on the water maze, food was made available *ad libitum* for 2 weeks and animals returned to their normal body weight.

All rats were handled once a week and checked for health problems (tumors). Body weight was recorded weekly and uterine weight was measured after sacrifice. Animal care and experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

Hormone treatment

For this chapter and all chapters regarding hormone replacement the following methods were used for hormone treatment. At 12–13 months, all subjects were anesthetized (4% isoflurane) and ovariectomized (OVX) via bilateral incisions. In accordance with animal care policy, animals were administered the analgesic, carprofen (0.05 mg/kg delivered intraperitoneally) prior to surgery and again 12 hours later. Following surgery, subjects were housed individually for 5 days to allow for recovery and then returned to pair- or triple-housed conditions. Hormone administration was initiated immediately after surgery. Animals were randomly assigned into the following five groups for the first cohort: no replacement (NR) (n = 3), chronic 17 β-estradiol (E_2) (n = 4), E_2 and MPA (E_2 + MPA) (n = 2), E_2 and progesterone (E_2 + P) (n = 4) or cyclic E_2 (CYE_2) (n = 2) and for the second cohort: no replacement (NR) (n = 8), chronic 17 β-estradiol (E_2) (n = 6), E_2 and MPA (E_2 + MPA) (n = 8), E and progesterone (E_2 + P) (n = 2) or cyclic E_2 (CYE_2) (n = 8).

17 β-estradiol (E_2) administration

All groups receiving hormones were given E_2 in their drinking water. Previous work from our lab has found that middle aged, acyclic intact females have circulating estrogen levels averaging 25–30 pg/ml (Markham & Juraska, 2002; Warren & Juraska, 2000). In a pilot study, we found that an E_2 dose of 70 μg/kg/day produced estrogen levels averaging 46 pg/ml. In order to keep the dose relatively low and in the physiological range for this age group we decreased the dose to 47 μg/kg/day. E_2 was first dissolved in 95% ethanol (2 mg/ml) and then dissolved in water as described in Gordon et al. (1986). Water consumption was checked weekly for each cage and remained between 60 and 80 ml/kg/day for each rat throughout the experiment for all groups. The dose of E_2 was calculated by taking the amount of water consumed by a cage and dividing by the sum of the weights in that cage. This value was then multiplied by the E_2 concentration in the water.

Chronic hormone treatment

The three chronic hormone treatment groups had E_2 in their drinking water every day: E_2, E_2 + P, and E_2 + MPA. On the day of OVX, one hormone pellet of either P or MPA was inserted in the appropriate groups through a small incision in the nape of the neck. The progesterone pellets were made from silastic tubing (Dow Corning) packed with crystalline hormone. Plugs were made using short wooden applicator sticks and the implants were sealed with silicone sealant, type A (Dow Corning). Studies have shown that 40 mm implants produce hormone levels approximating the peak circulating levels of progesterone achieved during the normal estrous cycle in the adult female rat (Hope, Bruns, & Thomas, 1992; M. S. Smith, Freeman, & Neill, 1975). The MPA pellets (1.5 mg) were purchased from Innovative Research of America. Using 1.5 mg 90 day release pellets results in a dose similar to those in women taking 2.5 mg per day

when expected daily release and average weight are factored in. Average weight for animals (.4 kg) was calculated at the start of the experiment and the average weight used for humans was 60 kg. Using these values the expected dose of MPA would be 41.7 micrograms/kg in rat and 41.6 micrograms/kg in humans. Both the progesterone and MPA pellets were replaced every 90 days. At the time of pellet replacement, all other groups received a sham surgery.

Cyclic hormone treatment

The group with cyclic E_2 treatment received E_2 in their drinking water three out of every 4 days. On the fourth day, the water did not contain E_2 and on the following day, the 4-day cycle was repeated. Due to a circadian drinking pattern, the highest elevation of estrogen levels occurs during peak nocturnal drinking periods and levels return to near baseline fifteen hours later (Gordon et al., 1986). Thus, it is likely that on the fourth day, when the animals were not receiving any E_2, levels of estrogen were not detectable.

Behavioral procedures

The first day of testing occurred after approximately 6 months of hormone treatment, so that subjects were 18–19 months old at the start of testing. Hormone treatment continued throughout the testing period.

Pretraining

Apparatus

The maze used during pretraining was a small wading pool (122 cm in diameter, 36 cm deep), located in a room other than the one used for testing. It had a sheet metal liner inserted to provide a vertical perimeter, and the visible escape platform (19 cm tall, 9 cm in diameter) was placed in the center of the pool. The pool was filled to 1 cm below the surface of the escape platform. The

temperature of the water was maintained at 26 °C ± 0.5 °C. The experimenter remained at one of four start locations around the pool for all pretraining trials.

Procedure

Pretraining began 2 days prior to the first day of testing. Subjects received four consecutive pretraining trials, each beginning from one of four start locations. Order of the start location was the same for all subjects. Rats were placed in the water while facing the edge of the pool and allowed 60 s to find the escape platform. If the platform was not located at the end of 60 s, the animal was guided to the platform by the experimenter. The subject remained on the platform for 15 s and was then removed from the maze and dried off using a towel. After completion of pretraining, rats were returned to their home cages.

Testing

Apparatus

The circular water maze testing tank (175 cm in diameter and 74 cm deep) was located in a room different than the one used for pretraining. A variety of extra-maze cues were present in the testing room. The maze was filled to a depth of 60 cm, 2 cm higher than the escape platform (58 cm tall and 10 square cm). White nontoxic paint was used to turn the water opaque, and the water was maintained at a temperature of 26 °C ± 0.5 °C.

Procedure

Subjects were tested across 4 days, with four trials per day (16 trials total). The location of the escape platform remained the same throughout the experiment. At the start of each trial, the subject was placed in the water, facing the edge of the tank, from one of four start locations. Start location order was the same for all animals. For each trial, the rat was given 60 s to find the platform, and the time to reach the platform was recorded. If the subject was unable to locate the

platform by the time 60 s had elapsed, the experimenter guided the subject to the platform. The subject remained on the platform for 15 s before being removed from the maze and dried off using a towel. The intertrial interval varied between 13 and 15 min. At the end of testing on Day 4, the platform was removed and a probe trial lasting 60 s was performed on the second cohort of animals. A video camera was mounted above the center of the tank and all trials were recorded. The experimenter recorded latency times with a stopwatch. After testing was complete, the pathlengths taken by the subjects to reach the platform were traced and then scanned into a computer and pathlengths were calculated using Image J (National Institutes of Health). Swim speed was calculated by dividing pathlength by latency.

Statistical analysis

Body weights and uterine weights were analyzed using a two-way ANOVA (5 Treatment × 2 Cohort). Pretraining latency was analyzed using a two-way (5 Treatment × 4 Trial) ANOVA with repeated measures on trial. For testing, latency and pathlength data were analyzed with each cohort of animals as a factor using a three-way (5 Treatment × 2 Cohort × 4 Day) ANOVA with repeated measures on day. Since there were no main effects of cohort and no cohort by treatment interaction, the two cohorts were then combined for analysis using a two-way (5 Treatment × 4 Day) ANOVA with repeated measures on day. Furthermore, post-hoc t-tests of treatment at each of the 4 days of testing were performed for both latency and pathlength because we expected differences between hormone groups in the later trials (Warren & Juraska, 2000). In addition, each day of testing was divided into two blocks of two trials for analysis because previous work in our lab indicated that differences in water maze performance may be due to the animals forgetting the task after a one-hour delay or overnight (Markham et al., 2002; Warren & Juraska, 1997). Therefore preplanned comparisons of Block 2 versus 3, 4 versus 5, and 6 versus 7 were

performed to compare performance at the beginning of each day with performance at the end of the previous day and determine whether animals forgot the task overnight. At the request of a reviewer, linear trend analyses were conducted to investigate whether the rate of improvement over trials differed between the groups. Swim speed during testing was analyzed using a two-way ANOVA (5 Treatment × 4 Day). Time spent in the target quadrant during the first half of the probe trial (30 s) and during the entire probe trial (60 s) was analyzed separately using a one-way ANOVA. Two-tailed t-tests were used for all post-hoc comparisons.

Results

Body weight and Uterine weight

There was a significant effect of treatment on body weight ($F(4, 41) = 4.258$, $p < 0.007$). Post-hoc t-tests revealed that the no replacement group weighed significantly more than all groups that received hormone treatment except the group receiving cyclic E_2 treatment for which there was a non-significant trend. (E_2: $p < 0.01$; E_2 + MPA: $p < 0.003$; E_2 + P: $p < 0.001$; CYE_2: $p < 0.08$). No other comparisons reached significance.

There was a significant effect of treatment ($F(4, 36) = 4.149$, $p < 0.01$) and cohort ($F(1, 36) = 11.169$, $p < 0.01$) on uterine weight but there was no treatment × cohort interaction ($F(4, 36) = 0.597$, $p = 0.667$). Post-hoc t-tests revealed that uterine weight in the no replacement group was significantly less than all groups that received hormone treatment except the group receiving chronic E_2 and progesterone treatment for which there was a trend. (E_2: $p < 0.01$; E_2 + MPA: $p < 0.01$; CYE_2: $p < 0.01$; E_2 + P: $p < 0.058$;), indicating that hormone treatment was physiologically effective (Table 1)

Pretraining

There was no significant effect of treatment on pretraining performance nor was there an interaction between treatment and pretraining trials (Figure 1).

Latency

There was a significant effect of day (F (3, 126) = 58.954, $p < 0.001$) as latency decreased as the animals became skilled at the water maze task. There was also a main effect of treatment (F (4, 42) = 2.683, $p < 0.05$). The treatment by day interaction was not significant. Post-hoc t-tests revealed that the chronic E_2 and MPA group performed significantly worse than all other groups receiving hormone treatment (E_2: $p < 0.007$; E_2 + P: $p < 0.03$; CYE_2: $p < 0.03$), but they were not significantly different from the no replacement group (Figure 2A). No hormone treated group was significantly different than the no replacement group. Analysis by 2-trial block indicated that none of the groups forgot the task overnight. At the suggestion of a reviewer we conducted a linear trend analysis of block to determine whether the rate of improvement over trials differed between the treatment groups. All groups improved performance across training as seen with the significant linear trend for block (F(1, 42) = 181.427, $p < 0.001$), but treatment groups did not differ in their rates of improvement.

Furthermore, preplanned comparisons of treatment at each of the 4 days of testing found that the animals receiving chronic E_2 had significantly shorter latencies than no replacement ($p < 0.05$) and cyclic E_2 ($p < 0.02$) treated animals on the last day of testing (Figure 3A). Importantly, there were no differences between any of the groups on the first day of testing.

Pathlength

Differences in pathlength were very similar to those found for latency. There was a significant effect of day on pathlength (F (3, 126) = 57.715, $p < 0.001$) such that pathlength became shorter

as testing progressed. There was also a main effect of treatment (F (4, 42) = 4.198, $p < 0.007$). The treatment by day interaction was not significant. Post-hoc t-tests revealed that the chronic E_2 and MPA group had significantly longer pathlengths than all other groups receiving hormone treatment (E_2: $p < 0.008$; E_2 + P: $p < 0.04$; CYE_2: $p < 0.004$), but they were not significantly different from the no replacement group (Figure 2B). No hormone treated group was significantly different than the no replacement group. Analysis by 2-trial block indicated that none of the groups forgot the task overnight except for E_2 + MPA between blocks 6 and 7 ($p < 0.04$). Similar to latency, all groups improved performance across training as seen with the significant linear trend for block (F(1, 42) = 137.055, $p < 0.001$), but treatment groups did not differ in their rates of improvement.

Furthermore, preplanned comparisons of treatment at each of the 4 days of testing found that on the last day of testing, the chronic E_2-treated animals had significantly shorter pathlengths than the cyclic E_2-treated animals ($p < 0.05$) and a trend in comparison to no replacement animals ($p < 0.09$) (Figure 3B). There were no differences between any of the groups on the first day of testing.

Swim speed

Swim speed decreased across the 4 days of training (F(3, 126) = 4.668, $p < 0.01$). There was no effect of treatment on swim speed, as well as no treatment by day interaction.

Probe trial

Treatment did not significantly influence the amount of time spent in the target quadrant during the full 60 s or during the first 30 s of the probe trial (Figure 4).

Discussion

The present study found that in middle aged female rats, long-term administration of chronic E_2 with MPA resulted in worse performance on the Morris water maze than all other types of hormone treatment but not the group with no replacement. Also by the fourth (and last) day of training, chronic E_2 treatment improved performance on the maze, but this effect was not seen earlier in training nor was it seen in the cyclic E_2 group. It should be noted that hormone treatment did not alter swim speed and there were no differences in pretraining latencies which suggests no motivational differences between groups.

The most striking finding is that middle aged rats receiving E_2 in combination with MPA, while not different than no replacement, performed significantly worse on the Morris water maze than all other groups receiving hormone treatment. To our knowledge, no published studies have investigated the effects of E_2 in combination with MPA, the most common progestin given to women, on cognition in an aging animal model. However, a recent study found that MPA administered without estrogen impaired performance on the water radial arm maze and the spatial water maze (Braden et al., 2010). In addition, the Women's Health Initiative found that conjugated equine estrogen in combination with MPA increased the number of subjects diagnosed with either probable dementia or mild cognitive impairment in post menopausal women (Espeland et al., 2004; S. R. Rapp et al., 2003; Shumaker et al., 2003; Shumaker et al., 2004). Although the dose of MPA was calculated to be similar to that in humans, the route of administration in humans is oral, while the route used in this study was subcutaneous. As previously noted, routes of administration alter rates of metabolism and therefore future studies need to assay levels of MPA in similarly treated animals of the same age.

The known neural effects of MPA are mixed. In vitro studies have found that E_2 protected against glutamate toxicity while E_2 in combination with MPA did not (Nilsen, Morales, & Brinton, 2006). In addition, progesterone protected against kainic acid-induced neuronal loss while MPA failed to do so (Ciriza et al., 2006). However, in male brain-injured rats, MPA resulted in a reduction of cerebral edema (Wright, Hoffman, Virmani, & Stein, 2008). Also, chronic treatment with conjugated equine estrogen alone or in combination with MPA resulted in an increase in the number of synapses in the hippocampus in young female rats (Silva, Mello, Freymuller, Haidar, & Baracat, 2000). At present, too little is known about long-term neural effects of hormone treatments during normal aging to predict behavioral outcomes. Furthermore, this task is a measure of spatial reference memory and it is possible that the outcome would be different in other tasks that depend on other types of memory (e.g., working, nonspatial), and this should thus be further evaluated.

Our finding that E_2 improved water maze performance on the fourth day of testing is consistent with previous work from our lab and others showing that chronic treatment with E_2 or estradiol benzoate benefited acquisition and retention of the water maze in middle age female rats (Foster et al., 2003; Markham et al., 2002; Talboom et al., 2008). Although we did not see significant differences on our probe trial, it is possible that the trends seen would have been significant with a larger sample size. Studies have also found that chronic E_2 treatment in middle aged OVX rats enhanced performance on the several other tasks including, the T-maze (Gibbs, 2000), object recognition (Vaucher et al., 2002), and spontaneous alternation (M. M. Miller et al., 1999). However, not all studies in middle aged animals have found beneficial effects of hormone treatment (Fernandez & Frick, 2004; Gresack & Frick, 2006). Since there is increasing evidence that hormone treatment may not be beneficial if initiated after long-term deprivation of hormones

all studies discussed previously initiated hormone treatment within 1 month of ovariectomy. Although, the length of hormone deprivation fails to explain the differing results of the previous studies, other variables to consider include the route of administration, the type of task used, and species and strain differences. In addition, a recent study found that reproductive experience alters responsiveness to estrogens (Barha & Galea, 2009), and it is possible that the use of retired breeders in the present study influenced our results. Furthermore, animals in the present study were exposed to a behavioral experiment prior to the present one which could have influenced performance.

The oral route of administration was used here to model the most common route of administration of hormone replacement in post menopausal women. In rodents, E_2 in the drinking water has been shown to produce levels that fluctuate throughout the day with the highest levels two hours after the onset of the dark cycle and then falling back to baseline during the following light cycle (Gordon et al., 1986). The only other study to use this route of administration found improved performance on object recognition but impaired reference memory on a water-escape motivated 8-arm radial arm maze (Fernandez & Frick, 2004). In contrast, another study from the same lab found that daily injections of E_2 did not improve performance on object recognition (Gresack & Frick, 2006). A possible explanation for the differing results is that the stress of injections may have interfered with the ability of E_2 to improve performance on object recognition. Chronic oral administration of hormones removes the stress associated with daily injections, thereby decreasing the possibility of an interaction of hormones and stress on the behavioral outcome. Furthermore, the oral route allows for first pass metabolism of the hormone which may affect the outcome of the treatment. Although we were not able to measure blood levels of estrogen, the group differences in body weights and uterine

weights indicate that the treatment was physiologically effective. Future studies should assay levels of estradiol (and MPA) in these treated groups. It should be noted that because estrogen was dissolved in ethanol, the hormone treated groups were exposed to a small amount of ethanol (0.014 g/kg/day) in their drinking water, while the no replacement animals were not. However, a previous study found no effect on the water maze when adult rats were exposed to 0.5 g/kg of ethanol, and ethanol levels of 2.0 g/kg were required for impairments on water maze performance in adult animals (Acheson, Ross, & Swartzwelder, 2001).

Unlike chronic E_2 treatment, there were no indications that cyclic E_2 treatment benefited performance on the Morris water maze. On the fourth day of testing, the chronically treated E_2 group performed significantly better than the animals treated cyclically. Animals in the cyclic E_2 group were on the third day of their 4-day cycle of hormone treatment and thus received E_2 on this day of testing. The studies that have investigated the effects of repeated cyclic hormone treatment are inconsistent. Weekly injections of E_2 plus progesterone enhanced performance of aged female rats on a delayed match-to-position T-maze task (Gibbs, 2000) and in aged rhesus monkeys, cyclic estradiol cypionate injections improved spatial working memory performance (P. R. Rapp et al., 2003). Two other studies have found that both continuous and intermittent E_2 significantly improved task acquisition in middle aged and aged animals (Bimonte-Nelson et al., 2006; Markowska & Savonenko, 2002). However, cyclic administration of estradiol benzoate did not improve performance in middle-aged female rats on a 12-arm radial arm maze (Ziegler & Gallagher, 2005). Furthermore, while continuous E_2 treatment had no effect on spatial working or reference memory in the radial arm maze, intermittent E_2 treatment impaired spatial reference memory (Gresack & Frick, 2006). Although the results of these studies differ, none of the studies found that cyclic treatment of hormones was more beneficial for task performance than chronic

treatment. It is important to note that most of the studies that have attempted to mimic the natural cycle of hormones have only simulated certain aspects of the cycle, so that the inconsistent results are not surprising. For example, estrogen cyclicity was mimicked in the current study, but not the fluctuation in progesterone levels. It is possible that more closely simulating the natural cycle by including fluctuations in both estrogen and progesterone levels may be more beneficial than the chronic treatment of ovarian hormones.

While E_2 in combination with progesterone did not benefit this task, it did not impair performance as did E_2 in combination with MPA. The few studies that have administered estrogens in combination with progesterone have resulted in equivocal findings. Some studies have found beneficial effects of estrogens given with progesterone. Gibbs (2000) found that weekly injections of E_2 in combination with progesterone improved T maze performance in aged rats. Furthermore, in middle aged rats, E_2 plus progesterone enhanced performance on the Morris water maze as well as E_2 alone (Markham et al., 2002). In contrast, another study found that progesterone reversed the beneficial effects of estradiol on this same task in middle aged rats (Bimonte-Nelson et al., 2006). However, similar to the present study, Bimonte-Nelson et al. (2006) found that while long-term treatment with estradiol and progesterone does not benefit the water maze, animals receiving this combination are not impaired on the task when compared to those not receiving hormone treatment.

Performance on the Morris water maze is dependant to a large degree on the hippocampus (Redish & Touretzky, 1998). Several studies have reported that estrogens influence the morphology and functional connectivity of the hippocampus in young adult females (Warren, Humphreys, Juraska, & Greenough, 1995; Woolley & McEwen, 1992; Woolley, Weiland, McEwen, & Schwartzkroin, 1997). However, E_2 treatment does not increase spines in aged

animals (Adams, Shah, Janssen, & Morrison, 2001). Interestingly, chronic MPA exposure results in an increase in synapses in the hippocampus of young animals (Silva et al., 2000), although it is not known if this occurs in aged animals. The neural mechanisms underlying the behavioral effects of hormone treatment may be different in young and aged animals. In future studies it will be imperative to examine the neural effects of MPA, the progestin most commonly prescribed to menopausal women, in an aging female model.

Figures and Tables

Figure 1.

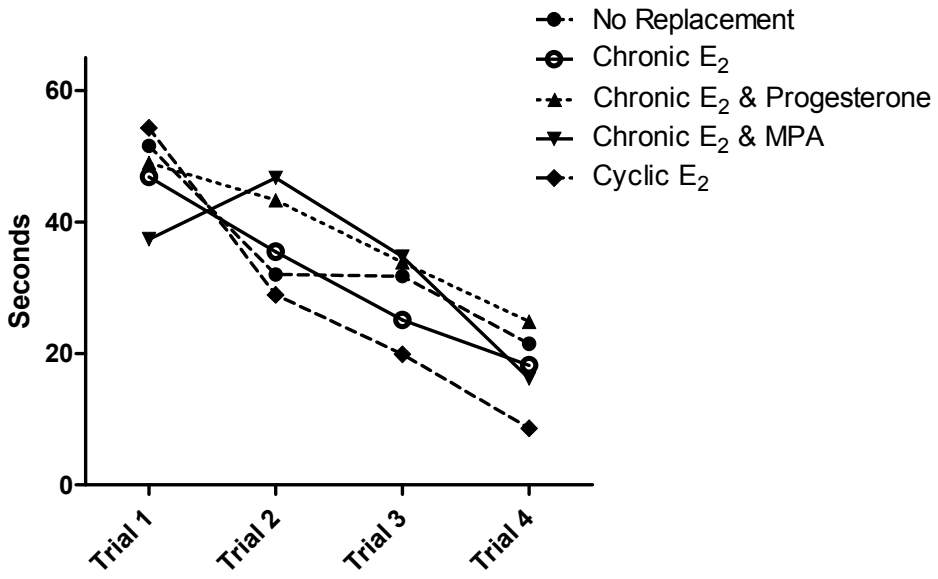

Figure 1. Mean latency to find the visible platform during four trials of pretraining. There were no significant differences between groups.

Figure 2.

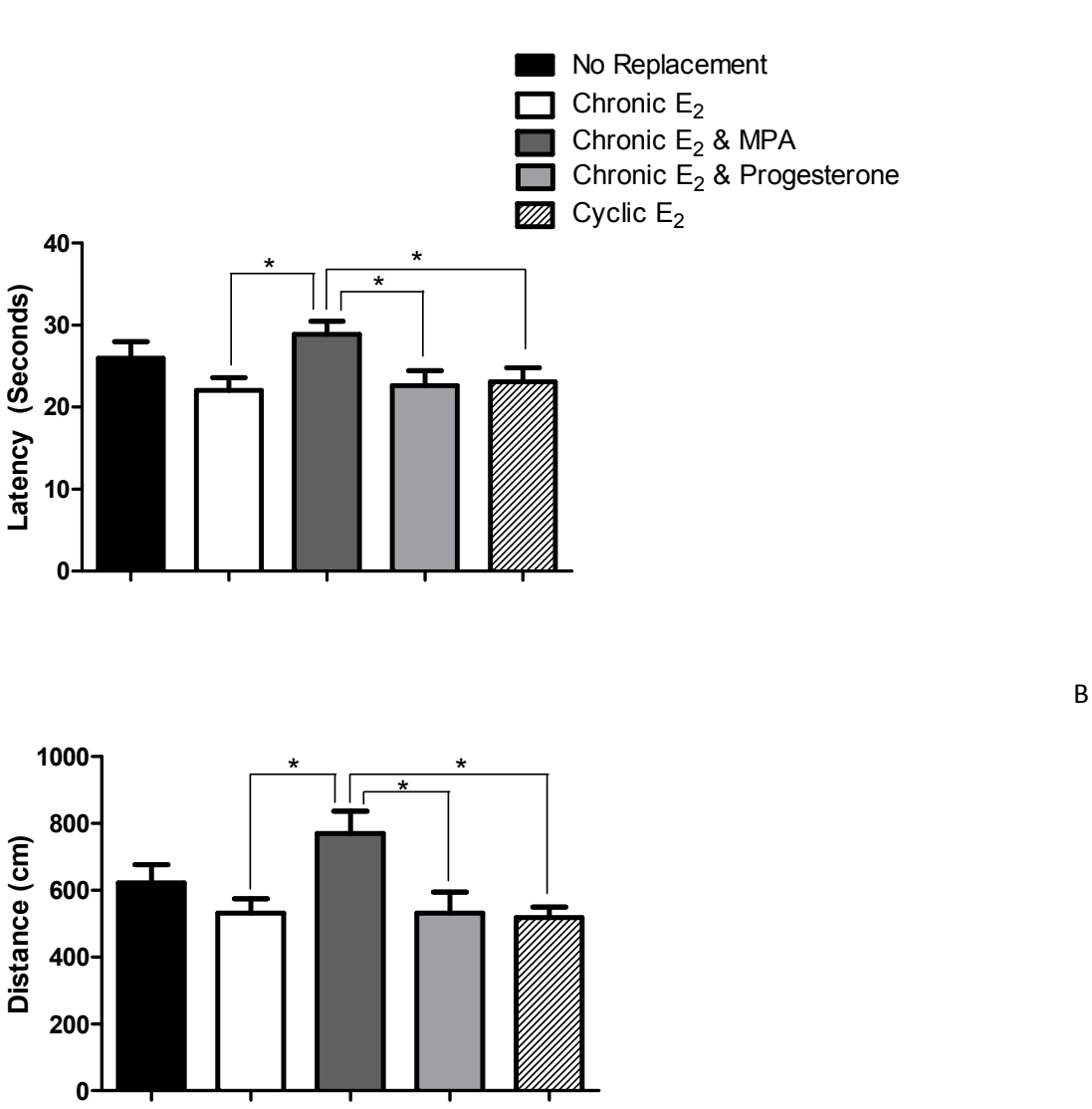

Figure 2. Mean ± SEM latency (A) and pathlength (B) to find the submerged platform averaged across four days of testing for all groups. The E + MPA group had significantly longer latencies (E: $p < .01$; E + P: $p < .03$; CYE: $p < .03$) and pathlengths (E: $p < .01$; E + P: $p < .04$; CYE: $p < .01$) than all other groups receiving hormone replacement.

Figure 3.

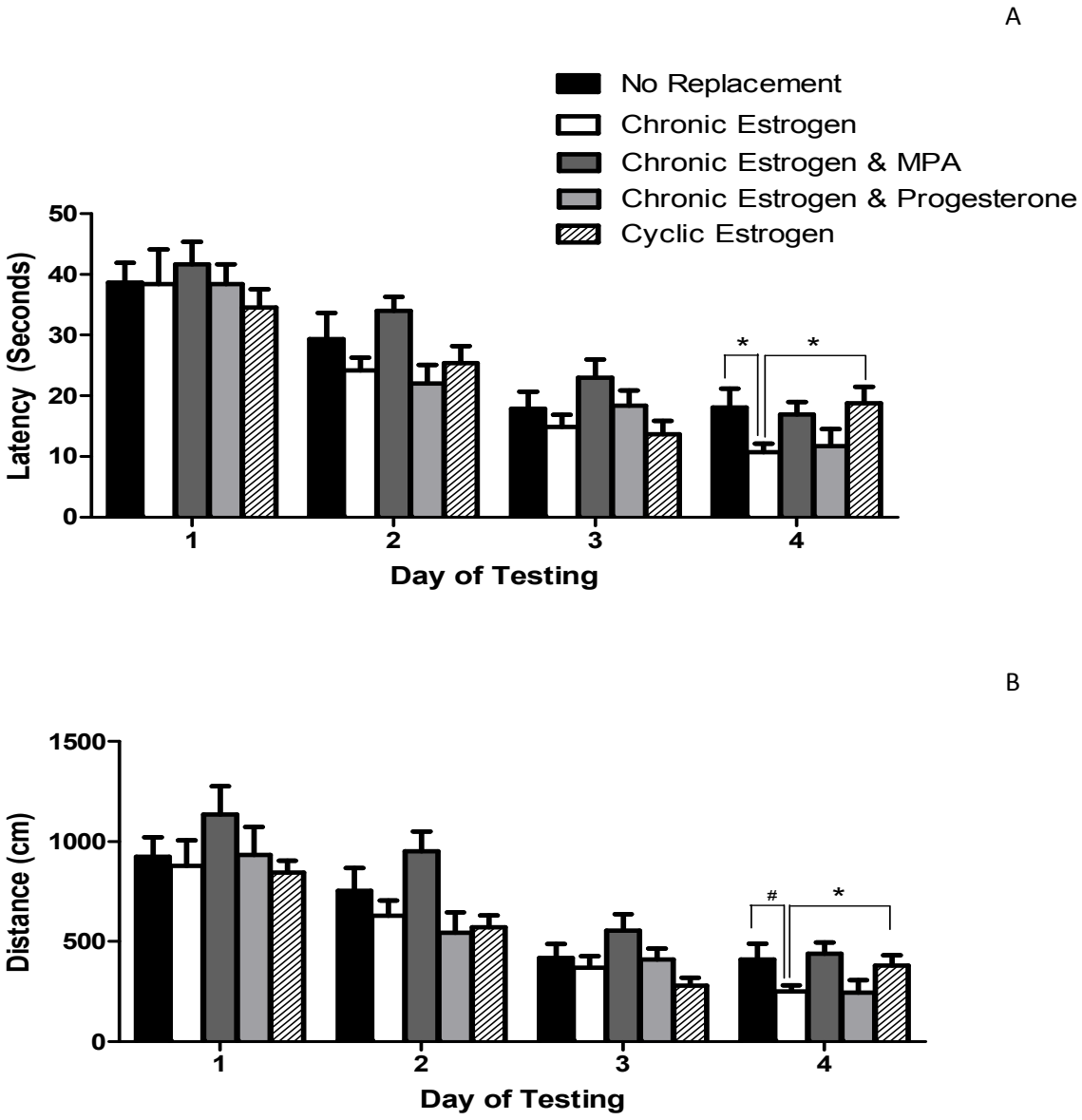

Figure 3. Mean ± SEM latency (A) and pathlength (B) to find the submerged platform for each of the four days of testing for all groups. The group receiving chronic 17 β-estradiol performed better on Day 4 than both NR and CYE groups. * ($p < .05$); # indicates a trend ($p < .09$).

Figure 4.

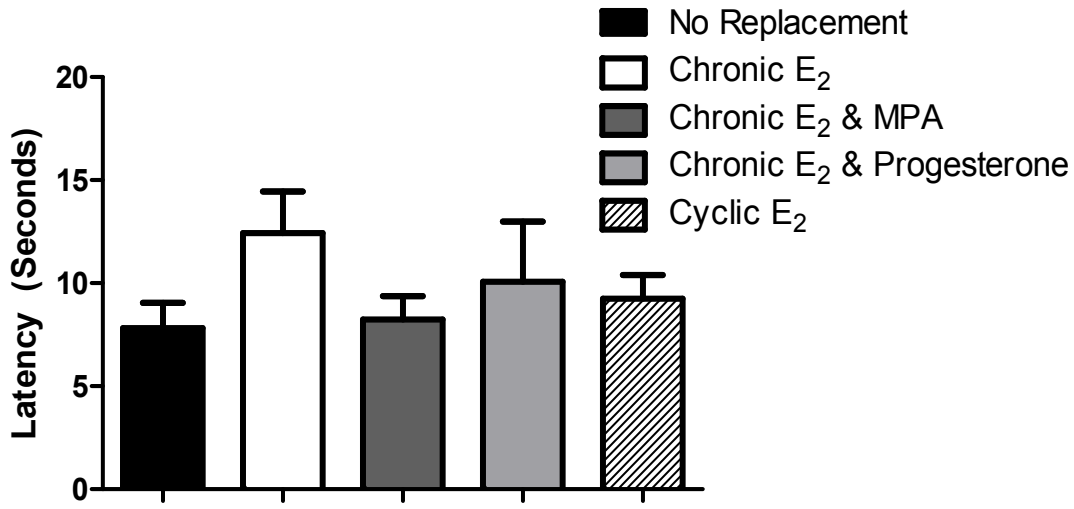

Figure 4. Mean ± SEM time spent in the target quadrant during the first thirty seconds of the probe trial for all groups. There were no significant differences between groups

Table 1. Body and Uterine Weight

Hormone Group	**Mean Body Weight (g)**	**Mean Uterine Weight (g)**
No replacement	584.5 ± 20.6	.1 ±.01
Chronic E_2	498.2 ± 22.2*	.17 ±.01*
Chronic E_2 & P	479 ± 32.4*	.15 ± .02#
Chronic E_2 & MPA	466.8 ± 27.6*	.17 ± .02*
Cyclic E_2	526.6 ± 23.4#	.16 ± .01*

Body and uterine weights were taken at sacrifice for all groups. No replacement animals weighed significantly more and had lower uterine weights that all hormone treated groups. * $p < .01$

CHAPTER 2

LONG-TERM REPLACEMENT OF ESTROGEN IN COMBINATION WITH MEDROXYPROGESTERONE ACETATE IMPROVES ACQUISITION OF AN ALTERNATION TASK IN MIDDLE AGED FEMALE RATS. [2]

[2] Previously published as: Chisholm NC, Juraska JM. Long term replacement of estrogen in combination with medroxyprogesterone acetate improves acquisition of an alternation task in middle aged female rats. *Behav Neurosci*. 2012 Feb;126(1):128-36

Abstract

Studies have shown that ovarian hormones protect against some of the cognitive deficits associated with aging. Although much of the literature in rodents has focused on hippocampal dependent tasks, studies suggest that tasks dependent on the prefrontal cortex are also influenced by ovarian hormones. The present study investigated the effects of ovarian hormone treatment during aging on a delayed alternation t-maze. Female Long Evans hooded rats were ovariectomized at middle age (11-12 months) and placed in one of 5 treatment groups: no replacement, chronic estradiol (E_2), cyclic E_2, chronic E_2 and progesterone, or chronic E_2 and medroxyprogesterone acetate (MPA). Following six months of hormone treatment, animals were trained to alternate in a t-maze. After reaching criterion, a series of delays from 5 to 90 seconds were introduced in random order. Rats receiving E_2 with MPA reached criterion significantly faster than animals not receiving treatment and those who received chronic or cyclic E_2 only. There was a nonsignificant trend for animals receiving E_2 and progesterone to reach criterion in fewer sessions than animals receiving E_2 only. Mode of administration, cyclic or chronic, did not affect performance. Hormones did not affect performance on the delayed alternation. This study, in combination with previous research, indicates that hormone effects cannot be generalized across tasks, age or duration, and long-term estrogen in combination with MPA can be beneficial for some tasks.

Introduction

In humans, several studies have identified the prefrontal cortex (PFC) as a region that has greater decline in gray matter volume during aging than other brain areas (Raz et al., 2005; Resnick et al., 2003). Also, the largest decline in metabolism during aging is localized to the medial prefrontal cortex (Pardo et al., 2007) and decreases in synaptic density, spine density, and dendritic arborization have been found in the aged human frontal cortex (de Brabander et al., 1998; Huttenlocher, 1979; Jacobs et al., 1997; Masliah, Mallory, Hansen, DeTeresa, & Terry, 1993). In addition to the anatomical changes, several studies have shown that the functions of the PFC, including behavioral flexibility, attention, memory, and language, are impaired during aging (reviewed in Stuss & Benson, 1984). For example, the aged have deficits in item recognition and source memory as well as delayed verbal recall (Wegesin & Stern, 2007). In addition, older adults performed worse on the Wisconsin card sorting task, a test of executive functioning, and on a free-recall task which was mediated more by executive functioning impairments than deficits in perceptual speed (Baudouin, Clarys, Vanneste, & Isingrini, 2009). The rat medial prefrontal cortex (mPFC) also experiences age-related changes. For example, reductions in both spine density and arborization of dendrites have been found in the mPFC of aged animals (Grill & Riddle, 2002; Markham & Juraska, 2002; Wallace et al., 2007) and the density of axospinous synapses decreases with age (Nakamura, Kobayashi, Ohashi, & Ando, 1999). Age-related changes also occur in the levels and turnover of dopamine and its metabolites (Godefroy, Bassant, Lamour, & Weil-Fugazza, 1991) as well as decreases in the amount of dopaminergic innervations (Mizoguchi et al., 2009), and NMDA receptor density (Castorina, Ambrosini, Pacific, Ramacci, & Angelucci, 1994; Magnusson & Cotman, 1993; Miyoshi, Kito, Doudou, & Nomoto, 1990). Similar to humans, the mPFC in rodents plays a role in many

cognitive abilities, including behavioral flexibility and memory for temporal order (Birrell & Brown, 2000; De Bruin et al., 2000; Kolb, 1990) and these measures are impaired during aging. For example, aged rats showed decreased performance, as compared to young rats, on an object recognition task (Wallace et al., 2007) and attentional set shifting (Barense, Fox, & Baxter, 2002). Also, studies have found deficits in aged animals when tested on delayed alternation tasks using the t-maze (Ando & Ohashi, 1991; Mizoguchi et al., 2009) and operant versions (Neese, Korol, Katzenellenbogen, & Schantz, 2010).

In both humans and rodents, several studies have shown that the presence of ovarian hormones may protect against some of these behavioral deficits. Although much of the literature has focused on hippocampal dependent tasks, ovarian hormones during aging also influence tasks dependent on the prefrontal cortex. In postmenopausal women, performance on a tasks relying on the prefrontal cortex, including digit ordering, delayed recall and a Wisconsin card sorting task, was improved after both short- term and long-term estrogen treatment (Krug et al., 2006; Wegesin & Stern, 2007). In addition, long-term estrogen only and estrogen and progestogen treatments enhance measures of verbal memory and improves performance on tasks of spatial and verbal working memory (Carlson & Sherwin, 1998; Duff & Hampson, 2000; Kampen & Sherwin, 1994). In rodents, chronic estradiol administered via injections and silastic capsules improves performance on a delayed-nonmatching-to-position task (Markowska & Savonenko, 2002; M. M. Miller et al., 1999) and long-term chronic estradiol alone or in combination with progesterone improves performance on a delayed-matching-to-position task (Gibbs, 2000). However, the Women's Health Initiative found that conjugated equine estrogen alone or administered with medroxyprogesterone acetate (MPA), the most common progestin given to women, failed to enhance cognition and increased the number of subjects diagnosed with either

probable dementia or mild cognitive impairment in post menopausal women (Espeland et al., 2004; S. R. Rapp et al., 2003; Shumaker et al., 2003; Shumaker et al., 2004). However, hormone treatment in the Women's Health Initiative was started several years after the onset of menopause and a recent review of the existing literature found hormone treatment to be beneficial when initiated close to the onset of menopause but not when initiated later (Rocca, Grossardt, & Shuster, 2011).

MPA is a synthetic analogue of progesterone and binds to progesterone receptors, as well as androgen and glucocorticoid receptors (Bamberger et al., 1999; Bardin et al., 1983). However, progesterone is readily metabolized to allopregnanolone (Majewska, Harrison, Schwartz, Barker, & Paul, 1986) and MPA inhibits the enzymes needed for this conversion (Jarrell, 1984; Lee, Miller, & Auchus, 1999; Penning, Sharp, & Krieger, 1985). Differences in mechanisms of action between these progestogens may result in divergent behavioral responses. Surprisingly, few studies have addressed the effects of MPA on cognition. Our lab has previously shown that long-term treatment with estrogen in combination with MPA attenuates the beneficial effects of estrogen alone or estrogen in combination with progesterone on water maze performance in middle aged rats (Lowry, Pardon, Yates, & Juraska, 2010). In addition, MPA administered without estrogen impaired performance on the water radial arm maze and the spatial water maze (Braden et al., 2010). When these two studies directly compared the behavioral effects of MPA with those of progesterone, they both found that progesterone did not impair performance on the water maze, but progesterone and MPA both impaired performance on the water radial arm maze (Braden et al., 2010; Lowry et al., 2010). Both of these studies used hippocampal dependant tasks and it is unknown how MPA would affect performance on tasks that are mediated by the mPFC.

Because tasks that are mediated by the mPFC are impaired during aging and because studies suggest that the presence of estrogen may prevent this, we chose to evaluate the effects of hormone treatment, including MPA, on age-related cognitive decline in the t-maze, a working memory task dependent on the mPFC. Middle aged female rats were ovariectomized, given ovarian hormone treatment, and subsequently tested on an alternation and delayed alternation task. We administered four different types of replacement: chronic replacement of estrogen alone, chronic replacement of estrogen and progesterone, chronic replacement of estrogen and MPA, or cyclic replacement of estrogen alone. Differences between groups of animals may suggest distinct roles for estrogen and progestogens during aging, and would indicate whether the effects vary with the type of progestogen administered. Whether treatment with estrogen in combination with MPA or progesterone will differentially affect performance on learned alternation or delayed alternation during aging is currently unknown.

Methods

Subjects

Subjects were 51 female Long Evans hooded rats purchased from Charles River Laboratories as retired breeders at the age of 11-12 months. Due to the large number of subjects and limited availability from the supplier, animals were run in three experimental cohorts. Animals from the same group were pair- or, if necessary, triple housed, in clear Plexiglass cages in a temperature-controlled environment on a 12:12-hr light–dark cycle. Food and water were available *ad libitum* to all animals, except during behavioral procedures during which the animals were maintained at 85-90% of their normal body weight. All rats were handled, weighed, and checked for health problems (tumors) once a week. At sacrifice, both body and uterine weights were measured. Animals from two of the cohorts were used in another behavioral experiment following

completion of the present task (Lowry et al., 2010). Animal care and experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

Hormone Treatment

The ovaries were removed from all subjects at 12-13 months. Subjects were anesthetized with 4% isoflurane and ovariectomized via bilateral incisions. Animals were administered the analgesic, carprofen (0.05 mg/kg delivered intraperitoneally), prior to surgery and again 12 hours later, in accordance with animal care policy. Subjects were housed individually for five days following surgery to allow for recovery and then returned to pair- or triple-housed conditions. Hormone administration was initiated the day of surgery and continued throughout the behavioral procedures. Animals were randomly assigned into the following five groups: no replacement (NR) (n = 12), chronic 17 β-estradiol (E_2) (n = 11), E_2 and MPA (E_2 + MPA) (n = 12), E_2 and progesterone (E_2 + P) (n = 7) or cyclic E_2 (n = 9).

17 β-estradiol (E_2) Administration. As in Lowry et al. (2010), all groups receiving hormones were given E_2 in their drinking water. Acyclic intact females continue to secrete estrogen at levels between 25–30 pg/ml and progesterone at 10-40 ng/ml depending on estropausal state (Markham & Juraska, 2002; A. E. Miller & Riegle, 1980; Warren & Juraska, 2000; Wise & Ratner, 1980). In a pilot study, we found that middle aged female rats drink a steady amount of water whether individually or group housed, and an aqueous E_2 dose of 70 µg/kg/day produced estrogen levels averaging 46 pg/ml. In order to keep the dose in the physiological range for this age group, we decreased the dose to 47 µg/kg/day. E_2 was first dissolved in 95% ethanol (2mg/ml) and then dissolved in water as described previously (Gordon et al., 1986). Water consumption was measured weekly for each cage and remained between 120-140 ml/kg/day

throughout the experiment for all groups. The dose of E_2 was calculated by taking the amount of water consumed by a cage and dividing by the sum of the weights in that cage. This value was then multiplied by the E_2 concentration in the water.

Chronic Hormone Treatment. Estradiol was available in the drinking water every day for the three chronic hormone treatment groups: E_2, E_2 + P, and E_2 + MPA. On the day of OVX, one hormone pellet of either P or MPA was inserted in the appropriate groups through a small incision in the nape of the neck. The progesterone pellets were made from silastic tubing (Dow Corning) packed with crystalline hormone. Plugs were made using short wooden applicator sticks and the implants were sealed with silicone sealant, type A (Dow Corning). Studies have shown that 40 mm implants produce hormone levels between those found in aging female rats in persistent estrus and persistent diestrus (J. W. Liu, Dawson, Peters, Baker, & Walker, 1997). The MPA pellets (1.5mg) were purchased from Innovative Research of America. Using 1.5mg 90 day release pellets results in a dose similar to those in women taking 2.5 mg per day when expected daily release and average weight are factored in. Progesterone and MPA pellets were replaced every 90 days. All other groups received sham surgeries at the time of pellet replacement.

Cyclic Hormone Treatment. E_2 was administered in the drinking water three out of every four days in the group receiving cyclic E_2 treatment. On the fourth day, the water did not contain E_2 and on the following day, the four day cycle was repeated. Due to a circadian drinking pattern, the highest elevation of estrogen levels occurs during peak nocturnal drinking periods and levels return to near baseline fifteen hours later (Gordon et al., 1986) which indicates that a day without E_2 resulted in levels near baseline.

Behavioral Procedures

The first day of testing occurred after approximately five months of hormone treatment, so that subjects were 17-18 months old at the start of testing. Hormone treatment continued throughout the testing period and all pellets were at approximately 55 days into their 90 day period. Food deprivation began five days prior to introduction to the maze. Animals were food deprived to 85-90% of their free feeding body weight.

Apparatus

The t-maze consisted of an approach alley (15 cm wide X 45 cm long) and two goal arms (15 cm wide X 45 cm long). The start box (15 cm wide and 26 cm long) opened into the approach alley. The walls of the maze were 15 cm high and were constructed of black plexiglass. Manually operated sliding doors were positioned at the entrance to each goal arm. The orientation and position of the maze were not changed during the experiment.

Testing Procedure

A previous study in our lab ran adolescent animals twice a day in order to complete testing within the adolescent time period (Koss, Franklin, & Juraska, 2011). We used the same procedure as that study; therefore in the present study, animals completed two sessions per day during all phases of the task.

Habituation. Animals were exposed to the maze during four 10-minute sessions over 2 days. During this time, each arm was baited with sunflower seeds and animals were allowed to roam the maze freely for 10 minutes.

Forced Alternation. Animals were placed in the maze for 10 forced alternation trials twice per day for three days for a total of 6 sessions. During each trial, the animal had access to only one open arm, which was baited with a sunflower seed reward and the opposite arm was blocked.

Once the animals had eaten the sunflower seed they were returned to the start box. The arm previously visited was then blocked, restricting access during the next run to only the alternate arm. For all trials, the animals remained in the chosen arm until they had eaten the sunflower seed or until 15 seconds had elapsed.

Alternation. On the first choice run, animals were placed in the start box and rewarded for choosing either arm. During the next 10 trials, animals were rewarded only when they entered the alternate arm. Each animal completed two sessions of 11 trials a day (1 free choice trial followed by 10 test trials) until a criterion of 80% over three consecutive sessions was achieved.

Delayed Alternation. All animals that reached criterion were then run for two sessions where they were introduced to short delays of 5, 10, and 15 seconds to reduce the level of stress associated with delays. Then the delayed alternation portion of the task was introduced. Delays of 5, 10, 15, 30, 60, 90 seconds were presented in a semi-randomized order. The procedures were the same as the alternation, except that after a trial, animals were returned to the start box where they would experience one of the delays. Each animal completed two sessions of 11 trials a day for a total of 15 sessions. This resulted in 25 trials at each delay. If an animal made an incorrect choice and then continued to choose the same arm, this was classified as a perseverative error in both the no-delay and delayed alternation portions of the task.

Post-behavior procedures. All animals were returned to *ad libitum* food. Two weeks after the completion of the present study, two of the three cohorts were run on a spatial water maze that has been previously reported (Lowry et al., 2010). At 19-20 months of age, they were sacrificed for neuroanatomical studies and body weight and uterine weights were recorded. Hormone treatment continued until sacrifice.

Statistical Analysis

Body weights and uterine weights were analyzed using a one-way ANOVA with cohort as a covariate. The number of sessions required to reach criterion was analyzed using a one-way ANOVA with cohort as a covariate. Performance on delayed alternation was analyzed using a two-way (5 Treatment x 6 Delay) ANOVA with repeated measures on delay and cohort as a covariate. Cohort was not significant for any of the analyses. Fisher's LSD tests were used for all post-hoc comparisons.

Results

Body Weight

There was a significant effect of treatment on body weight ($F(4, 45) = 6.470$, $p < .001$). Post-hoc tests revealed that the no replacement group weighed significantly more than all groups that received hormone treatment (E_2: $p < .001$; E_2 + MPA: $p < .001$; E_2 + P: $p < .01$; Cyclic E_2: $p < .02$) (Table 2).

Uterine Weight

There was a significant effect of treatment ($F(4, 45) = 5.902$, $p < .001$) on uterine weight. Post-hoc tests revealed that uterine weight in the no replacement group was significantly lower than all groups that received hormone treatment (E_2: $p < .001$; E_2 + P: $p < .01$; E_2 + MPA: $p < .001$; Cyclic E_2: $p < .03$), indicating that hormone treatment was physiologically effective (Table 2).

Alternation

A one way ANOVA revealed a main effect of treatment on the number of sessions to reach criterion ($F(4, 45) = 2.731$, $p < .05$). Post-hoc Fisher's LSD revealed that the group receiving E_2 + MPA required significantly fewer sessions to reach criterion than animals in three groups: no replacement ($p < .04$), chronic E_2 ($p < .02$), and cyclic E_2 ($p < .02$). There were non-significant

trends for animals that received E_2 + P to require fewer days to meet criterion than animals receiving chronic E_2 ($p < .09$) and cyclic E_2 ($p < .08$) (Figure 5). The groups did not differ in the number of perseveration errors during alternation.

Delayed Alternation

One animal that had met criterion in the learned alternation task failed to leave the start box during the delayed alternation task and was not included in analysis. There was a significant effect of delay ($F(5, 220) = 3.135$ $p < .01$) (Figure 6) such that increasing the intertrial delay significantly reduced performance in all treatment groups. Hormone treatment was not significant ($F(4, 44) = .959$ $p = .440$), nor was there a significant interaction between treatment and delay. The groups did not differ in the number of perseveration errors during delayed alternation.

Cyclic E_2

No effects were found within the cyclic E_2 group between days when E_2 was administered in the drinking water and when it was not.

Discussion

Long-term chronic administration of E_2 in combination with MPA enhanced acquisition of an alternation task in middle aged female rats. Animals receiving E_2 in combination with MPA reached criterion in fewer sessions than controls and animals who received chronic or cyclic E_2 only. Performance on delayed alternation decreased as the intertrial delay increased; however, hormone treatment failed to alter performance on the delayed portion of the task. These results are in contrast to our previous study in which 80% of the animals used in the present study were tested on the water maze and estradiol in combination with MPA impaired performance as compared to other hormone treated groups (Lowry et al., 2010).

Lesions of several different brain regions, including the hippocampus, striatum and prefrontal cortex, impair alternation behavior (reviewed in Lalonde, 2002) so that identifying a single brain region responsible for the behavioral outcome in this study is difficult. Furthermore, although animals can use allocentric spatial relationships to alternate in the t-maze, a study that manipulated several aspects of the maze environment found that performance on this task does not rely exclusively on these extramaze cues (Dudchenko, 2001). The beneficial effect of E_2 in combination with MPA found in the present study differs from previous studies examining the effects of MPA on cognition. Chronic MPA alone or in combination with E_2 impaired performance on the water maze in middle aged animals (Braden et al., 2010; Lowry et al., 2010). Performance on the water maze relies heavily on the hippocampus, which suggests that MPA might impair functioning of this brain region. Although MPA seems to impair hippocampal functioning, there is no evidence to suggest that this is true for other brain regions which may become engaged while solving the task used in the current study.

Interestingly, E_2 in combination with progesterone resulted in a more variable but similar pattern of fewer trials to acquire the alternation task as E_2 in combination with MPA. In agreement with this finding, weekly injections of E_2 in combination with progesterone improved t-maze performance in aged rats (Gibbs, 2000). It is unknown if progesterone and MPA have similar neural effects because few studies have directly compared them. Braden et al. (2010) found that chronic MPA significantly and progesterone marginally decreased levels of glutamic acid decarboxylase in the hippocampus when administered via osmotic pumps However, a single injection of progesterone transiently down regulated mRNA expression of $GABA_a$ subunits, where as MPA did not (Pazol, Northcutt, Patisaul, Wallen, & Wilson, 2009). If MPA and progesterone result in different neural outcomes, they may differentially affect cognition. Indeed

our previous study found that while long-term E_2 in combination with MPA impaired performance when compared to other hormone treated groups on the water maze, long-term E_2 in combination with progesterone did not (Lowry et al., 2010). However, the current study found similar effects of the two progestogens, emphasizing the importance of the task used to evaluate learning and memory.

The t-maze task used in the present study requires the animal to recall information that varies from trial to trial and therefore tests working memory (Markowska, Olton, & Givens, 1995). This study is the first to examine the effects of long-term treatment of E_2 in combination with MPA on working memory. Two previous studies have found that chronic MPA administered alone impairs working memory on the water radial arm maze (Braden et al., 2010; Braden et al., 2011). However, hormone treatment therapies contain MPA in combination with estrogen and the cognitive effects of these hormones may differ when administered alone. Although the present study found that E_2 in combination with MPA improved performance on a working memory task, future studies should examine if this generalizes to other working memory tasks. Interestingly, long-term chronic oral E_2 failed to alter performance on this task. Similar to our findings, chronic E_2 administered both orally and via daily injections did not affect the number of working memory errors in the radial arm maze (Fernandez & Frick, 2004; Gresack & Frick, 2006), and although chronic treatment with E_2 minipellets decreased the number of reference memory errors in the radial arm maze there was no effect on working memory errors (Heikkinen et al., 2004). Studies using the operant versions of the delayed spatial alternation task consistently find that E_2 administered chronically via silastic capsules during aging impairs performance (Neese et al., 2010; Wang, Neese, Korol, & Schantz, 2009) . Lesion studies indicate that the operant version of this task relies heavily on the mPFC (Sloan, Good, & Dunnett, 2006),

whereas performance on maze versions is altered by lesioning several different brain regions (Lalonde, 2002), indicating a fundamental difference between operant and maze versions. However, some studies have found improved performance after E_2 treatment on working memory tasks. Long-term chronic E_2 benefited acquisition of a delayed matching to position task, but similar to the present study, did not influence performance during delays (Gibbs, 2000). In addition, silastic capsules containing E_2 improved performance on a spontaneous alternation task in aged mice (M. M. Miller et al., 1999). Importantly, the duration of hormone treatment in Miller et al. (1 month) differed greatly from the present study in which hormones were administered for approximately six months prior to behavioral testing which might explain the differences between these two studies.

There is somewhat more agreement among studies evaluating the effects of estrogens on reference memory during aging. Several studies have found that E_2 or estradiol benzoate treatment in both middle aged and aged female rodents improves performance on the Morris water maze (Foster et al., 2003; Frick et al., 2002; Frye et al., 2005; Harburger et al., 2007; Lowry et al., 2010; Markham et al., 2002). It has become apparent that it is often not possible to generalize hormonal effects across different tasks and varying modes and lengths of administration (reviewed in Frick, 2009; J. M. Juraska & Rubinow, 2008).

Most studies evaluating the neural effects of hormone treatments have administered hormones acutely. Acute E_2 interacts with neurotransmitters systems, increases synaptophysin in the hippocampus, and increases neurotrophins in the entorhinal cortex (Bimonte-Nelson, Nelson, & Granholm, 2004; Frick et al., 2002; Gibbs, 1999). However, the few studies that have examined the neural outcomes of chronic long-term hormone treatment have found that changes observed with acute treatment return to baseline after chronic exposure. Chronic long-term treatment with

E_2 down-regulates estrogen receptors (Brown, Scherz, Hochberg, & MacLusky, 1996), and levels of choline acetyltransferase increase after acute E_2 treatment but return to normal after chronic treatment in the basal forebrain (Gibbs, 1997). Furthermore, acute treatment with estrogen and progesterone resulted in decreases in GABA receptor subunit expression 12 hours, but not 24 hours after treatment (Pazol et al., 2009). Therefore long-term chronic hormone treatment during aging might lead to a down regulation of estrogen receptors in cognitive brain regions and different behavioral outcomes than short term treatments.

It has been suggested that hormone treatments that more closely mimic the natural cycle of hormones might be more beneficial for cognition (Frick, 2009). The present study found that E_2 administered cyclically resulted in a similar rate of acquisition to E_2 administered chronically. Similar to the present study, continuous E_2 treatment had no effect on spatial working or reference memory in the radial arm maze, while intermittent E_2 treatment impaired spatial reference memory (Gresack & Frick, 2006). Cyclic administration of estradiol benzoate failed to improve performance on a 12-arm radial arm maze in middle aged female rats (Ziegler & Gallagher, 2005). However, weekly injections of E_2 plus progesterone enhanced performance of aged female rats on a delayed match-to-position T-maze task (Gibbs, 2000) and in aged rhesus monkeys, cyclic estradiol cypionate injections improved spatial working memory performance (P. R. Rapp et al., 2003). Continuous and intermittent E_2 improved task acquisition in middle aged and aged animals (Bimonte-Nelson et al., 2006; Markowska & Savonenko, 2002). Although these studies found beneficial effects of cyclic treatment as compared to controls, none of the studies found that cyclic treatment was more beneficial for task performance than chronic treatment. However, most of these studies only simulated certain aspects of the cycle. For example, estrogen cyclicity was mimicked in the current study, but not the fluctuation in

progesterone levels. More closely simulating the natural cycle by including fluctuations in both estrogen and progesterone levels may be more beneficial than the chronic treatment of ovarian hormones.

The route of administration used in a study can influence the outcome. Oral administration is the most common route of administration of hormone treatment in post menopausal women. Animal studies more commonly use injections or silastic capsules to deliver hormone treatments. These routes of administration have different pharmacokinetics than oral administration and do not allow for first pass metabolism of the hormone which may result in different outcomes of hormone treatment. Indeed, the only other study that has administered estradiol orally and examined effects on cognition in an animal model found improved performance on object recognition(Fernandez & Frick, 2004), while another study from the same lab found that daily injections of estradiol did not improve performance on the same task (Gresack & Frick, 2006). In the present study estradiol was administered orally both to model human treatment and to dose animals both cyclically and chronically. Although blood levels of estrogen are not available, group differences in body and uterine weights indicate that the treatment was physiologically effective, and preliminary results from a neuroanatomical study indicate treatment effects on brain structure (Packard, A.P., Lowry, N.C., Koss W.A., Juraska J.M., 2011).

After the Women's Health Initiative found that hormone treatment in postmenopausal women failed to enhance cognition and increased the number of subjects diagnosed with either probable dementia or mild cognitive impairment (Espeland et al., 2004; S. R. Rapp et al., 2003; Shumaker et al., 2003; Shumaker et al., 2004), researchers suggested that the presence of MPA in these hormone treatments might be responsible. However the current study found that E_2 in combination with MPA is beneficial for certain tasks. Taken together with our previous studies,

these data emphasize the importance of avoiding generalizations between tasks, age, and length of replacement as well as time without estrogen. Elucidating the neural effects of long-term hormone treatments might help elucidate what determines the behavioral outcome.

Figures and Tables

Figure 5.

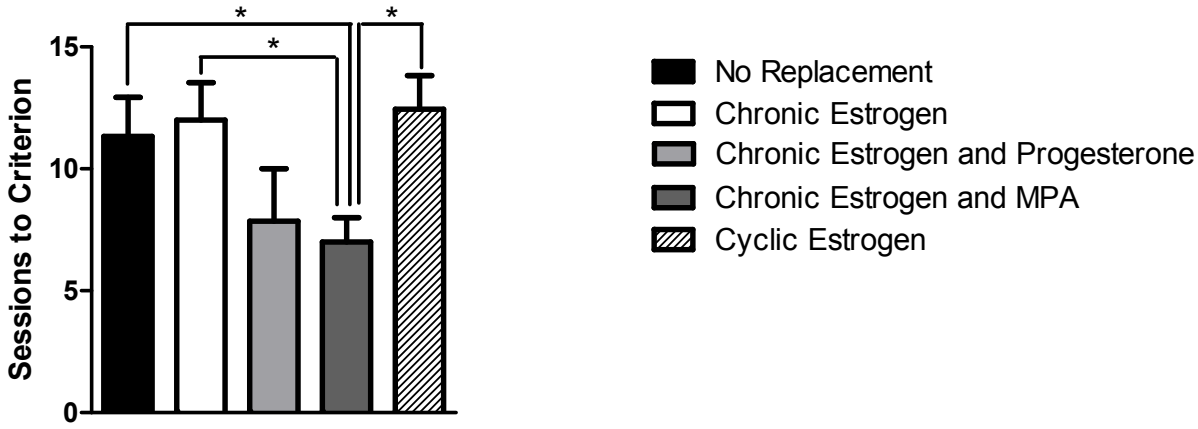

Figure 5. Mean ± SEM number of sessions to criterion for no delay alternation trials. Animals receiving E_2 + MPA required fewer sessions to reach criterion than animals in three groups: no replacement ($p < .04$), chronic E_2 ($p < .02$), and cyclic E_2 ($p < .02$). There were non-significant trends for animals that received E_2 + P to require fewer days to meet criterion than animals receiving chronic E_2 ($p < .09$) and cyclic E_2 ($p < .08$). * = $p<.05$

Figure 6.

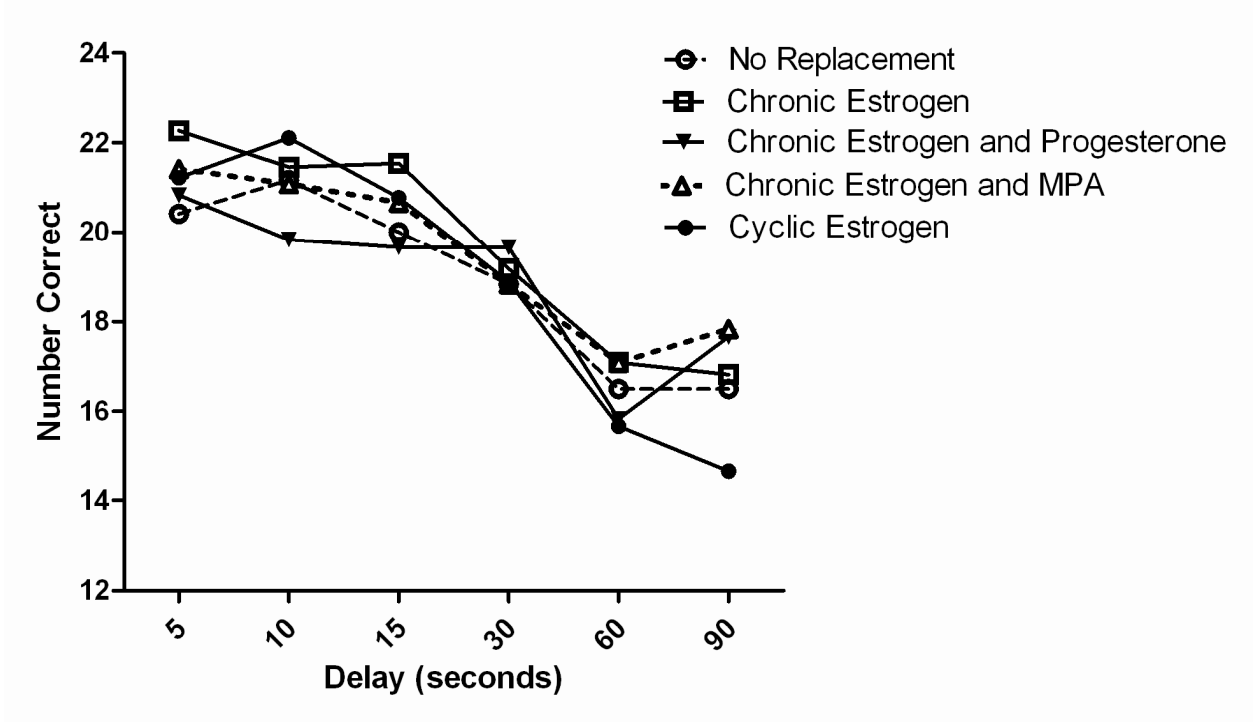

Figure 6. Mean number correct at each alternation delay. There was a significant effect of Delay ($p < .01$) such that increasing the intertrial delay significantly reduced performance in all treatment groups. Hormone treatment was not significant.

Table 2. Body and Uterine Weight

Hormone Group	Mean Body Weight (g)	Mean Uterine Weight
No replacement	573.1 ± 24.4	.09 ±.01
Chronic E_2	469.8 ± 22.5*	.15 ±.02*
Chronic E_2 & P	470.6 ± 19.93*	.13 ± .01*
Chronic E_2 & MPA	466.8 ± 27.6*	.16 ± .02*
Cyclic E_2	526.6 ± 23.4*	.16 ± .01*

(* = p< .05 compared to No replacement)

Body and uterine weights were taken at sacrifice for all groups. No replacement animals weighed significantly more and had lower uterine weights that all hormone treated groups. * p <.01

CHAPTER 3

EFFECTS OF LONG-TERM TREATMENT WITH ESTROGEN AND MEDROXYPROGESTERONE ACETATE ON SYNAPSE NUMBER IN THE MEDIAL PREFRONTAL CORTEX OF AGED FEMALE RATS.[3]

[3] Previously published as: Chisholm NC & Juraska JM. Effects of long-term treatment with estrogen and medroxyprogesterone acetate on synapse number in the medial prefrontal cortex of aged female rats. *Menopause*. In press.

Abstract

The present study investigated the effects of long-term hormone treatment, including the most commonly prescribed progestin, medroxyprogesterone acetate, during aging on synaptophysin labeled boutons, a marker of synapses, in the medial prefrontal cortex (mPFC) of rats. Female Long Evans hooded rats were ovariectomized at middle age (12-13 months) and were placed in one of 4 groups: no replacement (n=5), 17 β-estradiol alone (n=6), estradiol and progesterone (n=7) or estradiol and medroxyprogesterone acetate (n=4). Estradiol was administered in the drinking water and progestogens were administered via subcutaneous pellets that were replaced every 90 days. Following seven months of hormone replacement, animals were sacrificed and brains were stained for synaptophysin, a membrane component of synaptic vesicles. The density of synaptophysin labeled boutons was quantified in the mPFC using unbiased stereology and multiplied by the volume of the mPFC to obtain total number. Animals receiving estradiol and medroxyprogesterone acetate had significantly more synaptophysin labeled boutons in the medial prefrontal cortex than animals not receiving replacement ($p<.03$) and those receiving estradiol and progesterone ($p<.02$). In addition, there was a non significant trend for animals receiving estradiol alone to have more synapses than those receiving estradiol and progesterone. This study is the first to examine the effects of estradiol and medroxyprogesterone acetate during rat aging on cortical synaptic number. Estradiol with medroxyprogesterone acetate, but not progesterone, resulted in a greater number of synapses in the mPFC during aging than no replacement.

Introduction

Menopause in humans is associated with a loss of ovarian hormones and this decline in estrogen and progesterone has been linked to several of the symptoms related to menopause. Hormone therapies including Premarin (conjugated equine estrogens; CEE) and Prempro (CEE in combination with medroxyprogesterone acetate; MPA), have been approved to alleviate these symptoms. In women with a uterus, MPA, a synthetic analogue of progesterone, is administered in combination with estrogen therapy to prevent endometrial hyperplasia (Whitehead et al., 1979). Along with alleviating some of the symptoms of menopause, hormone treatment has beneficial effects on cognition (Joffe et al., 2006; Krug et al., 2006; LeBlanc et al., 2001). However, results from the Women's Health Initiative indicated that CEE alone or CEE administered with MPA results in an increased risk of stroke and dementia (Anderson et al., 2004; Shumaker et al., 2003; Wassertheil-Smoller et al., 2003). There is evidence that the timing of hormone replacement onset is an important factor and may explain the negative findings of the Women's Health Initiative studies (Daniel & Bohacek, 2010; Gibbs, 2000; Sherwin, 2009). Although data on the cognitive effects of hormone treatment in post-menopausal women seem inconsistent, several neurobiological studies have found that ovarian hormones increase synapses in the hippocampus of both young adult rats and non human primates. Ovariectomy decreased synapses in the CA1 region of the hippocampus (Gould, Woolley, Frankfurt, & McEwen, 1990) and estradiol administration increased the density of spines and synapses in the CA1 of young rats and non human primates (Adams et al., 2001; Choi et al., 2003; Leranth, Shanabrough, & Redmond, 2002; Woolley & McEwen, 1993). Chronic treatment with CEE increased synaptic density in the CA1 of young adult rats (Silva et al., 2000; Silva, Mello, Freymuller, Haidar, & Baracat, 2003). The synaptic response to estrogen is thought to involve estrogen receptor (ER)α

and ERβ, but the percentage of ERα labeled synapses (Adams et al., 2002) and the amount of ERβ (Yamaguchi-Shima & Yuri, 2007) decreases in the hippocampus during aging indicating that the aged brain might respond differently to estradiol. Indeed, estradiol increased synaptophysin, a membrane component of synaptic vesicles, in the CA1 of young animals but not in middle-aged rats (Williams et al., 2011). Although estradiol increased NR1, a subunit of the NMDA receptor, in the hippocampus of aged animals, there was no increase in synapse number (Adams et al., 2001).

Fewer studies have looked at the effects of hormone treatment on the prefrontal cortex (PFC), which exhibits greater neuroanatomical loss in both humans and other animals during aging than the hippocampus (J. M. Juraska & Lowry, 2011). Several studies have identified the human PFC as a region that has greater decline in gray matter volume during aging than other brain areas (Raz et al., 2005; Resnick et al., 2003). This change in volume is accompanied by age-related losses of dendrites and spines (de Brabander et al., 1998; Jacobs et al., 1997). There are also age-related losses of dendrites and spines in the PFC of non-human primates (Cupp & Uemura, 1980; Duan et al., 2003) and rats (Grill & Riddle, 2002; Markham & Juraska, 2002; Wallace et al., 2007). Similar to the hippocampus, estrogen can alter the structure of the PFC. Ovariectomy decreased spine density in the young adult rat PFC (Wallace et al., 2006) and estradiol benzoate increased spine synapse density in the PFC of young non-human primates (Leranth et al., 2008). Furthermore, long-term cyclic estradiol treatment increased dendritic spine density in the PFC of both young and aged rhesus monkeys (Hao et al., 2006; Tang et al., 2004), indicating that estradiol may affect the number of synapses in the aged PFC. In addition, intact females lose fewer spines during aging in the mPFC than males which may be due to the continued secretion

of estrogen and progesterone during estropause in rats (Markham & Juraska, 2002). To our knowledge, no study has examined the effects of progestogens on the PFC.

Progestogens can alter the effects of estrogen on behavior in aging females (Lowry et al., 2010; Warren & Juraska, 2000) and may also change the effect of estrogen on synaptic numbers. The few studies that have investigated the effects of progestogens on synapse number have examined the hippocampus. Although progesterone administered alone increased the density of synaptophysin in young adult rhesus monkeys, progesterone administered with estrogen resulted in densities similar to ovariectomized controls (Choi et al., 2003). A more recent study found that estradiol in combination with progesterone increased synaptophysin in hippocampal CA1 of young rats but not middle-aged or aged animals (Williams et al., 2011). MPA is the progestin most commonly prescribed to women and very little is known about the neural effects of long-term use. Both MPA and progesterone bind with high affinity to the progesterone receptor as well to the androgen and glucocorticoid receptors (Bamberger et al., 1999; Bardin et al., 1983; Sitruk-Ware, 2004). However, progesterone is readily metabolized to allopregnanolone (Majewska et al., 1986) whereas MPA inhibits the enzymes needed for this conversion (Jarrell, 1984; Lee et al., 1999; Penning et al., 1985). Differences in mechanisms of action between these progestogens may result in divergent neural responses. Surprisingly only one study has evaluated the effects of MPA on synapse number. Chronic treatment with MPA alone or in combination with CEE resulted in an increase in the number of synapses in the hippocampus of young rats (Silva et al., 2000). It is currently unknown if long-term treatment with MPA alters synapse number in the aged brain.

The potential effects of estrogen and the addition of progestogens on the number of synapses in the PFC are especially pertinent, given the dramatic changes in the PFC during aging. Therefore,

the present study examined the effects of long-term chronic hormone treatment on the number of synaptophysin labeled boutons in the mPFC of aging females.

Methods

Subjects

Subjects were 22 female Long Evans hooded rats purchased from Charles River Laboratories as retired breeders at the age of 11-12 months. Due to limited availability from the supplier, animals were run in two experimental cohorts. Animals from the same group were pair- or triple- housed, in clear Plexiglass cages in a temperature-controlled environment on a 12:12-hr light–dark cycle. Animals from these cohorts were used in two behavioral experiments prior to sacrifice (Chisholm & Juraska, 2012; Lowry et al., 2010). Standard rodent chow (Harlan 8604 Tekland) and water were available *ad libitum* to all animals, except during behavioral procedures during which the animals were maintained at 85-90% of their normal body weight. All rats were handled, checked for health problems (tumors), and weighed weekly. At sacrifice, both body and uterine weight were measured. Animal care and experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

Hormone Treatment

All subjects were ovariectomized (OVX) at 12-13 months, because intact female rats continue to secrete low levels of ovarian hormones during aging (Markham & Juraska, 2002; Warren & Juraska, 2000; Wise & Ratner, 1980). Subjects were anesthetized with 4% isoflurane and ovaries were removed via bilateral incisions. Animals were administered the analgesic, carprofen (0.05 mg/kg delivered intraperitoneally) prior to surgery and again 12 hours later, in accordance with animal care policy. Subjects were housed individually for five days following surgery to

allow for recovery and then returned to pair- or triple-housed conditions. Hormone administration was initiated the day of surgery and continued until sacrifice. Animals were randomly assigned into the following four groups: no replacement (n = 5), 17 β-estradiol (E_2) (n = 6), E_2 and MPA (E_2 + MPA) (n = 4), E_2 and progesterone (E_2 + P) (n = 7).

17 β-estradiol (E_2) Administration. As in Lowry et al. (2010), all groups receiving estradiol were given E_2 in their drinking water. A pilot study indicated that an E_2 dose of 47ug/kg/day produced estrogen levels in the physiological range for this age group (25-30 pg/ml) (Markham & Juraska, 2002; Warren & Juraska, 2000). E_2 was first dissolved in 95% ethanol (2mg/ml) and then dissolved in water as described in Gordon et al. (1986). Water consumption was measured weekly for each cage and remained between 60-80 ml/kg/day throughout the experiment for all groups. This range in water consumption resulted in E_2 doses between 40-55 ug/kg/day The dose of E_2 was calculated by taking the amount of water consumed by a cage and dividing by the sum of the weights in that cage. This value was then multiplied by the E_2 concentration in the water. Water bottles were filled with new estrogen water every third day and stock estrogen water was stored in a dark refrigerator.

Progestogen Treatment. On the day of OVX, one hormone pellet of either progesterone or MPA was inserted through a small incision in the nape of the neck in the appropriate groups. Progesterone pellets were made from silastic tubing (Dow Corning) packed with crystalline hormone. Studies have shown that 40 mm implants produce hormone levels between those found in aging female rats in persistent estrus and persistent diestrus (J. W. Liu et al., 1997).The MPA pellets (1.5mg) were purchased from Innovative Research of America. The 1.5mg 90-day release pellets result in a dose similar to that in women taking 2.5 mg per day when expected daily

release and average body weight are factored in. Progesterone and MPA pellets were replaced every 90 days. All other groups received sham surgeries at the time of pellet replacement.

Histology

At 19-20 months, after approximately 7 months of hormone treatment, rats were deeply anesthetized with sodium pentobarbital (2 mg/kg of a 50 mg/ml solution) and perfused intracardially with phosphate buffered saline followed with a solution of 4% paraformaldehyde, 4% sucrose and 1.4% sodium cacodylate in dH_2O. The brains were removed and stored in the same solution for 24 hours. Brains were then transferred to a sodium cacodylate buffer solution and shipped at room temperature to Neuroscience Associates (Knoxville, TN) for sectioning. Briefly, brains were cryoprotected in a glycerol and DMSO-based formulation prior to sectioning. Fixed brains from each cohort were embedded together in a gelatin block that was frozen-sectioned at 30μm. Every tenth section was stained for synaptophysin, a membrane component of synaptic vesicles, and other sections were saved. Adjacent sections were stained with methylene blue/azure II, a cell body stain, in our laboratory for volume calculations.

Volume estimation

Using cytoarchitectonic criteria (Krettek & Price, 1977; Van Eden & Uylings, 1985) the ventral mPFC (prelimbic (PL) and infralimbic (IL)) regions were parcellated at 31.25× using a camera lucida on coded slides stained with methylene blue/azure II. The ventral mPFC was parcellated starting with the first section containing white matter continuing through the first section in which the genu of the corpus callosum appeared. This resulted in parcellation of both hemispheres in four to five sections per subject. Parcellation criteria used for the ventral mPFC have been described in Markham et al. (2007). For the present study, the PL and IL were not separated. The border between PL and dorsal anterior cingulate (Acd) is marked by a broadening

of layer V and an increase in the density of layer 3 cells in the Acd as compared to the PL (Figure 7). Layers 2/3 and 5/6 were measured separately (rat mPFC lacks layer 4). Camera lucida tracings were scanned into a computer and Image J (version 1.44, 2010) was used to measure the area of each parcellation. The volume was then calculated by multiplying this area by the mounted tissue thickness between sections. Mounted tissue thickness was measured by determining the difference between the focal depth of the top and bottom of the tissue using the StereoInvestigator software program (MicroBrightField). Ten measurements of thickness were taken per animal. An average section thickness was calculated per animal and used in the calculations for that animal. Mounted section thickness was equivalent among all groups. Randomly, animals were selected for re-parcellation and the area of the ventral mPFC was recalculated. This was done to insure consistency in parcellation criterion. Area measurements remained within 5% between parcellation drawings for a given animal.

Synapse Number

Synaptophysin boutons were quantified in the PL and IL of the mPFC using the StereoInvestigator software program (MicroBrightField). The optical disector was used to obtain stereologically unbiased counts of synaptophysin density in each layer of the mPFC (Figure 8). Using this program, contours were drawn of layers 2/3 and layers 5/6 in the ventral mPFC. Both hemispheres from two sections containing the mPFC were used for counts. At least 200 synaptophysin boutons were counted within each layer (2/3, 5/6) for each subject. The area of the counting frame used was 4 μm × 4 μm with approximately 20 counting sites per section in both layers 2/3 and layers 5/6. Section thickness was used for dissector height excluding the .5 μm guard zones. Section thickness was measured at every fifth site on counted sections. Boutons fully inside the counting frame or those that contact the 'inclusion' line without

contacting the 'exclusion' line were included in counts (Figure 8). Average counts for each layer were divided by the volume of the counting frame to get the density of synaptophysin boutons. This density was then multiplied by the volume of the mPFC to obtain synapse number.

Statistical Analysis

Body weights, uterine weights, volume of the mPFC, and the total number of synaptic boutons, as well as those in layers 2/3 and in layers 5/6, were each analyzed using a one-way ANOVA with cohort as a covariate. Fisher's LSD tests were used for all post-hoc comparisons.

Results

Body and Uterine Weights

The ANOVA revealed a significant effect of treatment on body weight ($p < .01$). Post-hoc Fisher's LSD revealed that the no replacement group weighed significantly more than all groups that received hormone treatment (E_2: $p < .01$; $E_2 + P$: $p < .01$; $E_2 + MPA$: $p < .01$). No other comparisons reached significance (Table 3.)

The ANOVA resulted in a significant effect of treatment on uterine weight ($p < .02$). Post-hoc Fisher's LSD revealed that uterine weight in the no replacement group was significantly lower than all groups that received hormone treatment, indicating that hormone treatment was physiologically effective (E_2: $p < .01$; $E_2 + P$: $p < .01$; $E_2 + MPA$: $p < .01$).

Synapse Number

Volume of the mPFC was not significantly different between any of the groups. There was an overall effect of hormone treatment on the total number of synaptophysin boutons in the mPFC ($p < .05$). Post-hoc tests revealed that animals receiving $E_2 + MPA$ had more synaptophysin boutons than those receiving no replacement ($p < .03$) and $E_2 + P$ ($p < .02$). There was a non significant trend for animals receiving estrogen alone to have more synaptophysin boutons than

those receiving E_2 + P ($p < .09$) (Figure 9). For layers 2/3, hormone treatment did not alter synapse number ($p = .17$) (Figure 10A). Analysis of layers 5/6 found a significant effect of hormone treatment on the number of synaptophysin labeled boutons ($p = .04$). Post-hoc tests revealed that animals receiving E_2 + MPA had more synaptophysin boutons that those receiving no replacement ($p < .02$) and E_2 + P ($p < .02$) (Figure 10B).

Discussion

Long-term treatment with estradiol in combination with MPA to middle aged female rats resulted in greater numbers of synapses, as indicated by synaptophysin labeled boutons, in the mPFC as compared to ovariectomized controls. This is in agreement with the only other study to evaluate the effects of MPA on synapse number which found that MPA alone or administered with CEE increased synapses in the CA1 of young adult rats (Silva et al., 2000). Also, we have preliminary data showing that animals receiving estradiol in combination with MPA cyclically have greater numbers of synapses than ovariectomized controls (J. M. Juraska & Lowry, 2011). Importantly, synapse number decreases during aging (Gibson, 1983; Huttenlocher, 1979) and several measures related to synapse number are altered by aging in the PFC. There are age-related losses of dendrites and spines in the PFC of humans (de Brabander et al., 1998; Jacobs et al., 1997), non human primates (Cupp & Uemura, 1980; Duan et al., 2003), and rats (Grill & Riddle, 2002; Markham & Juraska, 2002; Wallace et al., 2007). These changes have been linked to age related cognitive decline. For example, during aging, non human primates experience a decrease in the density of axospinal synapses in the PFC which correlates with acquisition of a delayed non-match to sample task (Dumitriu et al., 2010). Furthermore, age related deficits on an object recognition memory task are associated with decreases in dendritic spine density in the mPFC of rats (Wallace et al., 2007). Because aging has been associated with a loss of synapses

and this loss has been linked to cognitive deficits, the greater number of synapses in the aged PFC following long-term exposure to estradiol and MPA could result in beneficial effects on behavioral tasks mediated by the mPFC.

However, few studies have evaluated the effects of estradiol in combination with MPA on cognition. MPA administered without estradiol impaired performance on the water radial arm maze and water maze (Braden et al., 2010; Braden et al., 2011). Although progesterone enhanced performance on the water maze and object recognition, MPA alone did not alter performance (Frye, Koonce, & Walf, 2010). A subset of the animals in the present study was tested on the water maze, and estradiol plus MPA resulted in impaired performance as compared to other hormone treated groups (Lowry et al., 2010). In contrast, when many of the same subjects were tested on a delayed alternation t-maze task, treatment with estradiol in combination with MPA resulted in animals requiring fewer sessions to reach criterion (Chisholm & Juraska, 2012). The brain region mediating performance on a task may play an important role in determining the behavioral outcome of hormone treatment, and the studies that have found that MPA impairs cognition have used tasks that rely heavily on the hippocampus (Braden et al., 2010; Braden et al., 2011; Lowry et al., 2010). The present study found that estradiol plus MPA results in more synaptophysin labeled boutons as compared with ovariectomized controls in the mPFC, suggesting that this combination of hormone treatment may be beneficial on tasks that rely more heavily on the mPFC.

Unlike estradiol and MPA, estradiol plus progesterone did not affect the number of synaptophysin labeled boutons. Although MPA is a synthetic analogue of progesterone, studies have found that these two progestogens do not share identical biological properties. MPA has a higher affinity for androgen and glucocorticoid receptors than progesterone (Bamberger &

Schulte, 2000) and progesterone is readily metabolized to allopregnanolone (Majewska et al., 1986) while MPA inhibits the enzymes needed for this conversion (Jarrell, 1984; Lee et al., 1999; Penning et al., 1985). Several studies have found that these two progestogens result in differential neural outcomes. For example, MPA, but not progesterone, suppresses cytokine production after an inflammatory stimulus in vitro (Bamberger et al., 1999). In addition, in vitro studies have found that progesterone protected against kainic acid-induced neuronal loss while MPA did not (Ciriza et al., 2006). Progesterone alone and in combination with estrogen protected against glutamate toxicity while MPA was not protective and prevented estradiol's influence on neuroprotection (Nilsen & Brinton, 2002a; Nilsen & Brinton, 2003). Also, treatment with estradiol and progesterone but not MPA, increased proliferation of neuroprogenitor cells in culture (L. Liu et al., 2010). Furthermore, progesterone increased levels of brain-derived neurotrophic factor while MPA decreased this measure (Jodhka, Kaur, Underwood, Lydon, & Singh, 2009). Although MPA and progesterone treatments without estrogen decreased levels of glutamic acid decarboxylase in the hippocampus, this decrease was only significant in those receiving MPA (Braden et al., 2010). Interestingly, most of these studies indicate a beneficial effect of progesterone on the measures evaluated, which was not found in the present study; however none of these studies examined synapse number or the importance of neuroprotection during normal aging. The current study administered hormones for approximately seven months in order to evaluate the long-term effects of hormone treatment and many of the previous studies have used acute treatments. Studies have found that chronic treatment of ovarian hormones results in different outcomes than more acute treatments (Gibbs, 1997; Morissette & Di Paolo, 1993; Pazol et al., 2009). It is possible that long-term hormone treatment results in receptors that are less sensitive and thus after several months of treatment

outcomes observed after acute treatment may return to baseline levels. It is unknown if long-term treatment with these two progestogens would affect receptors in a similar manner. Furthermore, previous studies comparing these progestogens have examined the effects on the hippocampus, and it is known that the anatomical structure of the PFC is particularly vulnerable to aging, while the hippocampus is not (reviewed in J. M. Juraska & Lowry, 2011). It is possible that because the hippocampus and mPFC respond differently to aging they are differentially affected by progestogens administered during this time.

Estrogen is known to alter several aspects of synaptic communication in young rats. For example, estrogen administered to ovariectomized animals, returns synaptophysin levels and spine densities in the CA1 to levels observed in intact controls (Gould et al., 1990; Williams et al., 2011; Woolley & McEwen, 1992). Also, ovariectomy decreases synaptophysin levels in the inner layer of the dentate gyrus and estrogen restores this (Stone, Rozovsky, Morgan, Anderson, & Finch, 1998). However, the aged hippocampus appears to be less responsive to the effects of estrogen. Estrogen treatment did not increase synapse density in the CA1 of aged animals (Adams et al., 2001) and there are decreased amounts of synaptic ERα immunoreactivity in this brain region (Adams et al., 2002). In addition, estradiol increased the amount of synaptophysin and opioid peptides in the CA1 and dentate gyrus of young animals, but did not alter the amount in aged animals (Williams et al., 2011). The present study found that long-term estrogen marginally increased synapses in the aged mPFC suggesting that in contrast to the hippocampus, the mPFC may remain responsive to estrogens during aging. This is in agreement with previous studies in non human primates. Long-term cyclic treatment with estradiol increased apical and basal dendritic spine density in the PFC of aged female rhesus monkeys and reversed age-related impairments on a delayed response task mediated by the PFC (Hao et al., 2006; P. R. Rapp et al.,

2003). It is important to note that the means in the present study were in the direction of estrogen treated animals having more synapses although it did not reach significance. Studies have found that rodent diets high in phytoestrogens result in a greater density of spines in both the hippocampus and PFC (V. Luine, Attalla, Mohan, Costa, & Frankfurt, 2006). As in most of the literature, animals in the present study were maintained on a standard rodent diet and it is possible that the low levels of soy in the diet increased the number of synaptophysin boutons in our no replacement animals minimizing differences between groups (Leranth et al., 2008). The differences that were found are especially notable in light of this possibility.

The effects of estrogen on synapse number have been shown to be mediated through estrogen receptors (F. Liu et al., 2008; Srivastava, Woolfrey, Liu, Brandon, & Penzes, 2010). However, because the effects of estrogen alone were subtle in the current study and the addition of MPA led to significantly more synapses as compared to no replacement, it seems likely that this effect was mediated by a different mechanism. Research suggests that synapse number is also regulated by IGF-1. IGF-1 null animals have decreased dendritic length and spines in the frontoparietal cortex (Cheng et al., 2003) and over expression of IGF-1increases the number of synapses in the dentate gyrus (O'Kusky, Ye, & D'Ercole, 2000). Importantly, IGF-1 protein levels are decreased during aging (Sonntag et al., 1999) and modulated by steroid hormones (Selvamani & Sohrabji, 2010a). Estrogen alone has been found to decrease IGF-1levels; however, estrogen administered with MPA resulted in an increase in IGF-1 in post-menopausal women (Malarkey et al., 1997) while estradiol administered with natural progesterone does not (Mozzanega et al., 2007). Therefore, the effects of estrogen in combination with MPA on synapse number seen in the current study may be mediated in part by increasing IGF-1 levels.

Given the vulnerability of the PFC to the effects of aging, the alteration of the mPFC by hormone treatment during aging may have implications for age-related cognitive deficits. Results from the current study indicate that the aged PFC remains responsive to certain hormone treatments and thus these treatments may protect against age-related cognitive deficits. Indeed, treatment with acute estrogen alone given to postmenopausal women benefited tasks relying on the PFC to a greater extent than those relying on the hippocampus (Krug et al., 2006). In addition, women not receiving estrogen replacement performed worse on several PFC dependant tasks than those receiving hormone treatment (Keenan et al., 2001). Future studies in humans should evaluate the effects of long-term hormone treatment, including the addition of a progestogen on PFC dependant tasks.

Conclusions

This study identifies the prefrontal cortex as a brain region that is altered by long-term chronic hormone treatment during aging in the rat. Specifically, treatment with estradiol and MPA resulted in more synaptophysin labeled boutons in the mPFC relative to females with no replacement or replacement with estradiol and progesterone. These findings provide insight into the neural effects of long-term hormone treatment.

Figures and Tables

Figure 7.

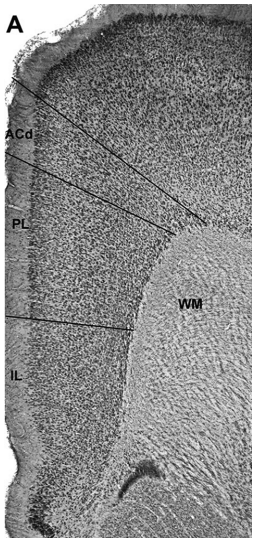

Figure 8.

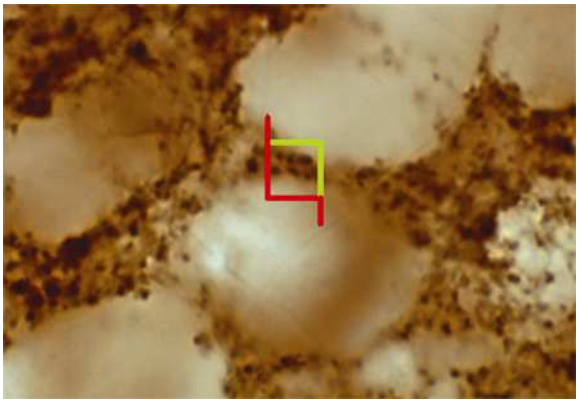

Figure 7. Coronal section through the medial prefrontal cortex with cell bodies stained using Methylene blue/Azure II. Borders of the prelimbic and infralimbic regions are shown based on the cytoarchitectonic characteristics revealed by this stain (Krettek & Price, 1977; Van Eden & Uylings, 1985). Reprinted from Markham et al. (Markham et al., 2007).

Figure 8. High magnification image of the mPFC stained for synaptophysin, a membrane component of synaptic vesicles. The counting frame used to stereologically quantify the number of boutons was 4 μm by 4 μm.

Figure 9.

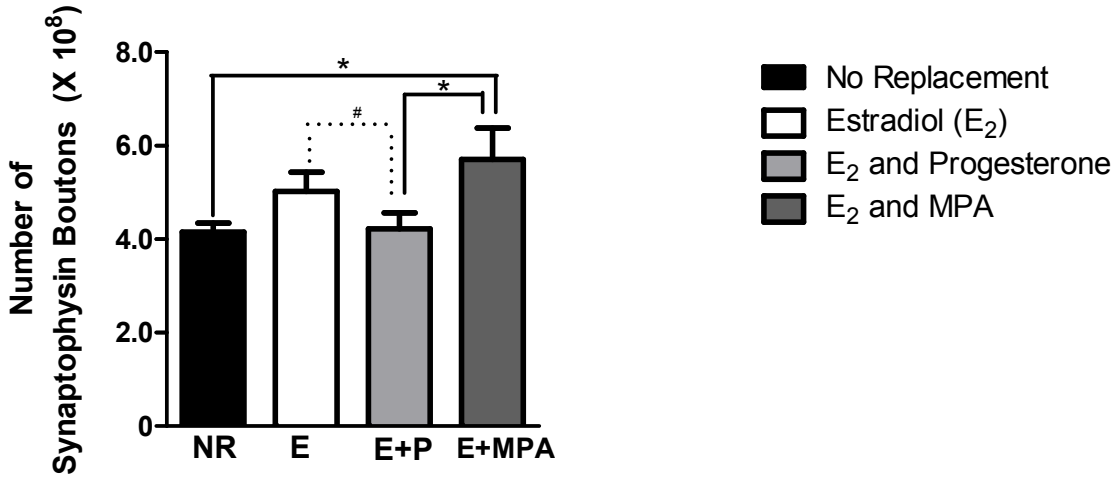

Figure 9. Total number (mean + SEM) of synaptophysin labeled synaptic boutons in the mPFC. Animals receiving E_2 + MPA had more synaptophysin boutons than those receiving no replacement and E_2 + P. There was a non significant trend for animals receiving estrogen alone to have more synaptophysin boutons than those receiving E_2 + P (*p <.03, #p < .09).

Figure 10.

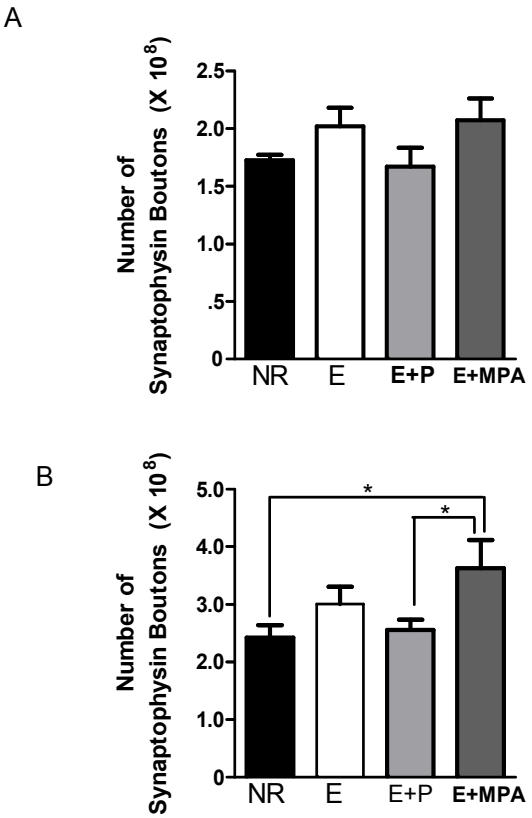

Figure 10. Total number (mean + SEM) of synaptophysin labeled synaptic boutons in Layers 2/3 of the mPFC. Hormone treatment did not significantly alter the number of synapses. B. Total number (mean + SEM) of synaptophysin labeled boutons in Layers 5/6 of the mPFC. Animals receiving E_2 + MPA had more synaptophysin labeled boutons that those receiving no replacement and E_2 + P (*$p < .02$).

Table 3. Body and Uterine Weight

Hormone Group	Mean Body Weight (g)	Mean Uterine Weight (g)
No replacement	619.6 ± 37.7	.05 ±.01
Estrogen	501.8 ± 35.0*	.12 ±.02*
Estrogen & P	472.7 ± 28.1*	.11 ± .01*
Estrogen & MPA	451.3 ± 66.2*	.12 ± .02*

Body and uterine weights were taken at sacrifice for all groups. No replacement animals weighed significantly more and had lower uterine weights that all hormone treated groups. * $p < .01$

CHAPTER 4

THE EFFECTS OF LONG-TERM TREATMENT WITH ESTRADIOL AND MEDROXYPROGESTERONE ACETATE ON TYROSINE HYDROXYLASE FIBERS AND NEURON NUMBER IN THE MEDIAL PREFRONTAL CORTEX OF AGED FEMALE RATS.[4]

[4] Submitted for publication as: Chisholm NC, Packard AR, Koss WA, Juraska JM. The effects of long-term treatment with estradiol and medroxyprogesterone acetate on tyrosine hydroxylase fibers and neuron number in the medial prefrontal cortex of aged female rats. Endocrinology

Abstract

Menopause in humans is associated with undesirable side effects and many women initiate hormone treatment therapies to alleviate these symptoms. Research suggests that these treatments may also affect cognition, and studies in young animals indicate that hormone treatment can alter several neuroanatomical measures. However, very little is known about the effects of long-term hormone treatment on the aging female brain. This study investigated the effects of hormone treatment on neuron number and tyrosine hydroxylase fibers in the rat medial prefrontal cortex (mPFC). Female Long Evans rats were ovariectomized at middle age (12-13 months) and placed in one of 4 groups: no replacement, estradiol (E_2), E_2 and progesterone, or E_2 and medroxyprogesterone acetate (MPA). At 20 months of age, animals were sacrificed and the brains were Nissl stained and immuno-stained for tyrosine hydroxylase. Neuron number and tyrosine hydroxylase fiber density were quantified in the mPFC. Hormone treatment did not alter neuron number. Treatment with E_2 and MPA resulted in greater tyrosine hydroxylase densities than no replacement animals in layer 1 and layers 2/3 ($p < .05$). In layers 2/3, animals receiving E_2 also had greater tyrosine hydroxylase densities than no replacement animals ($p < .01$). These results indicate that long-term hormone treatments alter dopaminergic fibers and potentially the functioning of the aging mPFC.

Introduction

Gonadal hormone levels decrease in both men and women during aging (reviewed in (Lamberts, van den Beld, & van der Lely, 1997); however aging females experience a dramatic decrease in ovarian hormones at the onset of menopause. Many women initiate hormone treatment therapies, including Premarin (conjugated equine estrogens; CEE) and Prempro (CEE in combination with medroxyprogesterone acetate; MPA), to alleviate the symptoms of menopause, and the presence of these hormones may alter the course of aging in females. Indeed, women using estrogen therapy during menopause have a greater gray matter density in superior frontal gyrus than non users (Lord, Engert, Lupien, & Pruessner, 2010) and non-users have lower gray matter concentration in orbitofrontal cortices than both estrogen users and young women (Robertson et al., 2009). However, the Women's Health Initiative found that CEE alone or CEE administered with MPA results in an increased risk of stroke and dementia (Anderson et al., 2004; Shumaker et al., 2003; Wassertheil-Smoller et al., 2003). More recent studies suggest that the timing of hormone replacement initiation may explain the negative findings of the Women's Health Initiative studies (Daniel & Bohacek, 2010; Gibbs, 2000; Sherwin, 2009).

The effects of hormone treatment on the prefrontal cortex are especially important given the changes that are occurring in this brain region during human aging. The prefrontal cortex has been identified as a region that has greater decline in gray matter volume during aging than other brain areas (Raz et al., 2005; Resnick, Pham, Kraut, Zonderman, & Davatzikos, 2003). Decreases in synaptic density, spine density, and dendritic arborization have also been found in the aged human frontal cortex (de Brabander, Kramers, & Uylings, 1998; Huttenlocher, 1979; Jacobs, Driscoll, & Schall, 1997; Masliah, Mallory, Hansen, DeTeresa, & Terry, 1993). In the mPFC of aged rats, reductions have been found in both spine density and arborization of

dendrites (Grill & Riddle, 2002; Markham & Juraska, 2002; Wallace, Frankfurt, Arellanos, Inagaki, & Luine, 2007). In addition, a loss of neurons has been observed in the aging human cortex overall (Pakkenberg & Gundersen, 1997) and in the PFC during aging in non-human primates and rodents (Smith, Rapp, McKay, Roberts, & Tuszynski, 2004; Yates, Markham, Anderson, Morris, & Juraska, 2008). However, there is evidence from our lab that this loss is sexually dimorphic with males, but not females, losing neurons during aging in the mPFC (Yates et al., 2008). The presence of low levels of ovarian hormones in rats after the cessation of the cycle (Dudley, 1982) may protect females from this age-related neuron loss, thus providing a possible mechanism by which hormone treatment decreases shrinkage associated with aging. There is extensive research demonstrating the neuroprotective properties of estradiol in culture or young animals (Garcia-Segura, Azcoitia, & DonCarlos, 2001); however, more recent work finds that estradiol is not neuroprotective in a middle-aged stroke model (Selvamani & Sohrabji, 2010a). In addition, estrogen and progesterone have been shown to have neuroprotective properties in vitro; whereas MPA counteracts the neuroprotective effects of estrogen (Nilsen & Brinton, 2002b). It is unknown how neuroprotection relates to normal aging and to date there have been no studies examining the effects of hormone treatment during aging on neuron number in the mPFC.

Ovarian hormones are known to alter several neuroanatomical measures including synapse number and spine density in the prefrontal cortex of young (Leranth, Hajszan, Szigeti-Buck, Bober, & MacLusky, 2008; Tang et al., 2004) and aged rhesus monkeys (Hao et al., 2006). In addition, a recent study from our lab found that long-term treatment with estradiol in combination with MPA during aging resulted in a greater number of synapses than no replacement animals (Chisholm & Juraska, in press). This alteration in synapse number could

result from several different cellular changes including preservation of neurotransmitter functioning. Dopamine is of particular interest because of the changes that are known to occur in this system during aging in the PFC. For example, dopamine receptors decrease during aging in humans, nonhuman primates and rodents (de Keyser, De Backer, Vauquelin, & Ebinger, 1990; Gozlan et al., 1990; Lai, Bowden, & Horita, 1987; Seeman et al., 1987), with the fastest rate of decline commonly found in the frontal cortex (Inoue et al., 2001; Kaasinen et al., 2000; Kaasinen et al., 2002). Dopaminergic functioning in the PFC is also altered by ovarian hormones. Levels of dopamine fluctuate in the prefrontal cortex across the estrous cycle in intact animals (Dazzi et al., 2007). Furthermore, acute estradiol and an ER β agonist, increased dopamine metabolites in the PFC of young adult rats (Inagaki, Gautreaux, & Luine, 2010; Jacome et al., 2010), and tyrosine hydroxylase (TH) immunoreactivity in the PFC of adult female monkeys is decreased after ovariectomy and restored with estradiol administered with progesterone (Kritzer & Kohama, 1998; Kritzer & Kohama, 1999). However the effects of ovarian hormones during aging on this neurotransmitter system have not been thoroughly investigated and it is possible that effects of hormone treatment may be different in young and aged animals.

The present study administered different combinations of long-term hormone treatments to aging female rats to determine the effects on both neuron number and dopaminergic fibers. Middle aged female rats were ovariectomized, and placed in one of four groups: no replacement, estrogen only, estrogen and progesterone, estrogen and MPA. Differences between groups of animals may suggest distinct roles for estrogen and progestagens during aging and provide insight into how hormone treatments may alter the course of normal aging.

Methods

Subjects

Subjects were female Long Evans hooded rats purchased from Charles River Laboratories as retired breeders at the age of 11-12 months. Due to limited availability from the supplier, animals were run in three experimental cohorts. Animals from all cohorts were included in the analysis of neuron number (n=41) and animals from cohorts 1 and 2 were included in the analysis of tyrosine hydroxylase density (n=21). Animals from the same group were pair- or triple- housed, in clear cages on a 12:12-hr light–dark cycle. Standard rodent chow (Harlan 8604 Tekland) and water were available *ad libitum* to all animals, except during behavioral procedures (Chisholm & Juraska, 2012) during which the animals were maintained at 85-90% of their normal body weight. All rats were handled, checked for health problems (tumors), and weighed weekly. Both body and uterine weight were measured at sacrifice. Animal care and experimental procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

Hormone Treatment

Subjects were ovariectomized (OVX) at 12-13 months under isoflurane anesthesia. Hormone administration was initiated the day of surgery and continued until sacrifice. Animals were randomly placed in one of the following four groups: no replacement (NR) (n = 12), 17 β-estradiol (E_2) (n = 12), E_2 and progesterone (E_2 + P) (n = 7) or E_2 and MPA (E_2 + MPA) (n = 10).

17 β-estradiol (E_2) Administration. As in Chisholm & Juraska (2011) all groups receiving estradiol were given E_2 in their drinking water. E_2 was first dissolved in 95% ethanol (2mg/ml) and then dissolved in water as described in Gordon et al.(1986). A pilot study found that an E_2 dose of 47μg/kg/day resulted in estrogen levels in the physiological range for this age group (25-

30 pg/ml) (Markham & Juraska, 2002; Warren & Juraska, 2000). Water consumption was measured weekly for each cage and remained between 60-80 ml/kg/day throughout the experiment for all groups. This resulted in E_2 doses between 40-55 μg/kg/day. The dose of E_2 was calculated by taking the amount of water consumed by a cage and dividing by the sum of the weights in that cage. This value was then multiplied by the E_2 concentration in the water.

Progestogen Treatment. At the time of OVX, one hormone pellet of either progesterone or MPA was inserted in the nape of the neck in the appropriate groups. Progesterone pellets were 40 mm in length and made from silastic tubing (Dow Corning) packed with crystalline hormone. Studies have shown that 40 mm implants produce hormone levels between those found in aging female rats in persistent estrus and persistent diestrus (J. W. Liu, Dawson, Peters, Baker, & Walker, 1997). MPA pellets (1.5mg) were purchased from Innovative Research of America. The 1.5mg 90-day release pellets result in a dose similar to that in women taking 2.5 mg per day when expected daily release and average body weight are factored in. Progesterone and MPA pellets were replaced every 90 days and all other groups received sham surgeries at the time of pellet replacement.

Histology

At approximately 20 months, after 8 months of hormone treatment, rats in cohorts 1 and 2 (n=21) were deeply anesthetized with sodium pentobarbital (2 mg/kg of a 50 mg/ml solution) and perfused intracardially with phosphate buffered saline followed with a solution of 4% paraformaldehyde, 4% sucrose and 1.4% sodium cacodylate in dH_2O. The brains were removed and stored in the same solution for 24 hours. Brains were then transferred to a sodium cacodylate buffer solution and shipped at room temperature to Neuroscience Associates

(Knoxville, TN) for sectioning. Unstained 30 μm sections were returned to our lab where they were stained with methylene blue/azure II, a cell body stain, and for tyrosine hydroxylase.

The third cohort of animals (n=20) were only used for neuron counts and were processed according to the following methods. All rats were deeply anesthetized with sodium pentobarbital (2 mg/kg of a 50 mg/ml solution), and intracardially perfused with Ringer's wash (2 minutes) followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and stored in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer for 21 days, followed by cryoprotection in 30% sucrose for three days. Brains were coronally sectioned and 60 μm thick sections were mounted then stained with methylene blue/azure II.

Immunohistochemistry

Sections from cohorts 1 and 2 (NR (n = 5); E_2 (n = 6); E_2 + MPA (n = 4); E_2 + P (n = 6)) were immunoreacted according to procedures found in Adler et al. (1999) with a few modifications. Sections were rinsed in 0.1 M PBS, pH 7.4 (3 X 15 minutes), incubated in 1% H_2O_2 in PBS for 30 min. Sections were then rinsed in Tris-buffered saline (TBS 3 X 15 minutes), pH 7.4, placed in blocking solution (TBS containing 10% normal swine serum; NSS) for 2 hours, and incubated in primary antiserum (diluted in TBS containing 1% NSS, two days, 4°C). Anti-TH antibodies (Chemicon International, Temecula, CA) were used at a working dilution of 1:1000. Following incubation in primary antibody, sections were rinsed in TBS, placed in biotinylated secondary antibodies (Vector, Burlingame, CA; 2 h room temperature; 1:100), rinsed in TBS, and then incubated in avidin–biotin-complexed horseradish peroxidase for two hours at room temperature (ABC; Vector, Burlingame, CA). Sections were then rinsed in TBS, pH 7.4 and reacted by using Sigma Fast Tabs (Sigma, Saint Louis, MO). Sections were

immediately mounted and allowed to dry overnight. The following day, slides were dehydrated and coverslipped.

Volume estimation

Using cytoarchitectonic criteria (Krettek & Price, 1977; Van Eden & Uylings, 1985) the ventral mPFC (prelimbic (PL) and infralimbic (IL)) regions were parcellated at 31.25× using a camera lucida on coded slides stained with methylene blue/azure II. The ventral mPFC was parcellated starting with the first section containing white matter continuing through the first section in which the genu of the corpus callosum appeared. This resulted in parcellation of both hemispheres in four to five sections per subject. Parcellation criteria used for the ventral mPFC have been described in Markham et al.(2007). Layers 2/3 and 5/6 were measured separately (rat mPFC lacks layer 4). Camera lucida tracings were scanned into a computer, and Image J (version 1.44, 2010) was used to measure the area of each parcellation. The volume was then calculated by multiplying this area by the mounted tissue thickness between sections. Mounted tissue thickness was measured by determining the difference between the focal depth of the top and bottom of the tissue using the StereoInvestigator software program (MicroBrightField, Chicago, IL). An average section thickness was calculated per animal and used in the calculations for that animal.

Neuron number estimation

Neurons were quantified in the PL and IL of the mPFC using the StereoInvestigator software program (MicroBrightField). The optical disector was used to obtain stereologically unbiased counts of cell density in each layer of the mPFC. Using this program, contours were drawn of layers 2/3 and layers 5/6 in the ventral mPFC and at least 200 neurons were counted within each layer (2/3, 5/6) for each subject. Section thickness was measured at every fourth site

on counted sections. Only neurons fully inside the counting frame or those that contact the 'inclusion' line without contacting the 'exclusion' line were included in counts. Average counts for each layer were divided by the volume of the counting frame to calculate the cell density. This number was then multiplied by the volume to obtain cell number.

Fiber Density

Examination of tyrosine hydroxylase immunoreactive fibers was carried out in two PFC-containing sections (300μm apart) of each brain. Z-stacked images were acquired using a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Thornwood, NY) and compressed using Axiovision software (Figure 11A). Within each layer of the PFC (1, 2/3, and 5/6), three pictures were taken (two in the PL one in the IL). A total of 18 pictures were taken per animal (9 per section).

Image J was used to measure image pixel density (the percent of the image in black) in two ways: first as a binary image, taking thickness into account (Figure 11B), and then as a skeletonized image, reducing the thickness to 1 pixel wide (Figure 11C). Skletonized images were analyzed because previous studies that have quantified tyrosine hydroxylase fibers used this type of image (Kritzer & Kohama, 1999); binary images were quantified to determine if fiber thickness might be altered. In addition, the binary images were mulitplied by the volume of the mPFC for each animal to test whether the total volume of TH fibers differed between groups.

Statistical analysis

Body and uterine weights were analyzed using a one-way ANOVA with cohort as a covariate. Neuron number was analyzed using a one-way ANOVA with cohort as a covariate for each layer separately and then with all layers combined for total neuron number in the mPFC. The total pixel density percentage and the total volume of TH immunoreactive fibers in both

binary and skeletonized images were analyzed using a one-way ANOVA for layer 1 and layers 2/3 separately. TH fibers were not analyzed in layers 5/6 due to the dark background in the images acquired. Fisher's LSD tests were used for all post hoc comparisons.

Results

Body and Uterine Weights

Hormone treatment significantly affected body weight in animals used for neuron number analysis ($F(3, 36) = 9.897$, $p < 0.01$). Post-hoc Fisher's LSD revealed that the no replacement group weighed significantly more than all groups that received hormone treatment (E_2: $p < .01$; $E_2 + P$: $p < .01$; $E_2 + MPA$: $p < .01$). No other comparisons reached significance (Table 4). Analysis of animals that were used only for tyrosine hydroxylase analysis also resulted in a significant effect of treatment on body weight.

Uterine weight was significantly altered by hormone treatment in animals used for neuron number analysis ($F(3, 36) = 11.197$, $p < 0.01$). Post-hoc Fisher's LSD revealed that uterine weight in the no replacement group was significantly lower than all groups that received hormone treatment, indicating that hormone treatment was physiologically effective (E_2: $p < .01$; $E_2 + P$: $p < .01$; $E_2 + MPA$: $p < .01$) (Table 4). There was also a significant effect of treatment on uterine weight in animals that were used only for tyrosine hydroxylase.

Neuron Number and Volume

Hormone treatment did not significantly alter volume or neuron number in any layer or in all layers combined of the mPFC (Figure 12).

Tyrosine Hydroxylase

Layer 1

Analysis of binary images in layer 1 found that hormone treatment significantly altered the total number of TH immunoreactive fibers ($F(3, 17) = 3.903$, $p < 0.03$) (Figure 13A). Post-hoc tests showed that animals receiving E_2+MPA had a higher density of TH immunoreactive fibers than those receiving no replacement ($p < 0.01$) and than those receiving E_2+P ($p < 0.02$). There was a trend for animals who received E_2 to have a higher TH fiber pixel density than animals who received no hormone replacement ($p = 0.06$). Analysis of the skeletonized images revealed a similar pattern within layer 1 ($F(3, 17) = 2.648$, $p < 0.09$) (Figure 13B). Post-hoc tests demonstrated that animals receiving E_2 +MPA had a significantly higher density of TH fibers than those receiving no replacement ($p < 0.03$) and a weak trend ($p < 0.08$) toward E_2 +MPA having higher pixel densities than E_2 +P (Figure 3B). There was a trend ($p = 0.06$) for E_2 animals to have a higher pixel density percentage than NR animals. There was an effect of hormone treatment on the volume of tyrosine hydroxylase fibers ($F(3, 16) = 4.286$, $p < 0.03$) and post-hoc tests found the same group differences as the analysis of fiber density (Figure 14A).

Layers 2/3

Analysis of the binary images found that hormone treatment significantly altered the TH immunoreactive fiber density within layers 2/3 ($F(3,15) = 3.387$, $p < 0.05$)(Figure 15A). Post-hoc tests demonstrated that animals receiving E_2 and E_2 +MPA had significantly higher TH fiber densities than no replacement animals ($p < 0.05$) (Figure 15A). There was a trend for animals receiving E_2 +P to have more TH fibers than no replacement ($p < .06$). Analysis of skeletonized images revealed a significant effect of hormone treatment on pixel density percentage of TH immunoreactive fibers in layers 2/3 $F(3, 15) = 6.496$, $p < 0.01$) (Figure 15B). Post-hoc analysis

found that all hormone treated groups had greater TH fiber density than no replacement animals (E_2 +MPA: $p = 0.02$; E_2 +P: $p < 0.05$; and E_2: $p = 0.001$). In addition, the E_2 only group had a significantly higher pixel density than the E_2 +P group ($p < 0.03$). There was also a main effect of hormone treatment on the volume of tyrosine hydroxylase fibers ($F(3, 14) = 3.721$, $p < 0.04$). Post-hoc tests found that animals receiving E_2+MPA and E_2 alone had significantly higher volumes of TH than no replacement animals ($p < .02$). There was a trend for animals receiving E_2 +P animals to have a greater volume of TH than no replacement animals ($p < 0.07$) (Figure 14B).

The volume of TH fibers and the number of synaptophysin labeled boutons in the mPFC found in our previous study (Chisholm & Juraska, in press) were examined using Pearson correlation analysis. In layers 2/3 the volume of TH fibers showed a positive correlation with the number of synaptophysin labeled boutons ($r = .44$, $p < .05$) (Figure 16).

Discussion

Long-term treatment of middle aged female rats with estradiol alone and in combination with MPA resulted in a greater density of tyrosine hydroxylase fibers in the mPFC as compared to ovariectomized controls. This is the first study to evaluate the effects of long-term estrogen treatment with or without MPA, on the prefrontal dopaminergic system during aging in females. However, these results are congruent with several studies in young animals that find that the prefrontal dopaminergic system is influenced by ovarian hormones. (Dazzi et al., 2007; Inagaki et al., 2010; Kritzer & Kohama, 1998; Kritzer & Kohama, 1999). Although no studies have looked at the prefrontal cortex during aging, the effects of acute estrogen treatment on the aged striatum have been examined. Acute estradiol benzoate increased basal dopamine levels in the striatum of both young and aged rats, while a similar treatment increased dopamine receptors in

the striatum of young females, but not middle aged females (McDermott, 1993; Roy, Sheinkop, & Wilson, 1982). The changes in the dopaminergic system found after long-term treatment with estradiol and MPA in the current study are also consistent with the greater number of synapses found in a previous study from our lab after the same treatment (Chisholm & Juraska, in press). Because dopamine fibers are known to decrease with normal aging in the prefrontal cortex (Mizoguchi, Shoji, Tanaka, Maruyama, & Tabira, 2009), it is most likely that this hormone treatment is preventing the normal loss associated with aging, rather than by causing new fibers to innervate the prefrontal cortex.

Interestingly, estradiol in combination with progesterone did not consistently result in a greater density of tyrosine hydroxylase fibers. In fact, in layer 1 of the mPFC, not only did estrogen with progesterone not alter tyrosine hydroxylase fibers compared to controls, it resulted in fewer fibers than estradiol in combination with MPA. This finding is in agreement with our previous study which found that long-term estradiol in combination with MPA resulted in a greater number of synapses, while estradiol with progesterone failed to alter synapse number (Chisholm & Juraska, in press). MPA is a synthetic analogue of progesterone; however these two progestogens do not share identical biological properties. For example, progesterone is metabolized to allopregnanolone (Majewska, Harrison, Schwartz, Barker, & Paul, 1986) while MPA inhibits the enzymes required for this conversion (Jarrell, 1984; Lee, Miller, & Auchus, 1999; Penning, Sharp, & Krieger, 1985) and MPA has a higher affinity for androgen and glucocorticoid receptors than progesterone (Bamberger & Schulte, 2000). These two progestogens often result in differential neural outcomes although most of these studies indicate a beneficial effect of progesterone on the measures evaluated. For example, progesterone alone and in combination with estrogen protected against glutamate toxicity while MPA was not

protective and prevented estradiol's influence on neuroprotection (Nilsen & Brinton, 2002a; Nilsen & Brinton, 2003). In addition, progesterone protected against kainic acid-induced neuronal loss in vitro whereas MPA did not (Ciriza, Carrero, Frye, & Garcia-Segura, 2006). Treatment with estradiol and progesterone but not MPA, increased proliferation of neuroprogenitor cells in culture (L. Liu et al., 2010). Furthermore, MPA decreased levels of brain-derived neurotrophic factor while progesterone increased this measure (Jodhka, Kaur, Underwood, Lydon, & Singh, 2009). MPA, but not progesterone, significantly decreased levels of glutamic acid decarboxylase in the hippocampus (Braden et al., 2010), and suppressed cytokine production after an inflammatory stimulus in vitro (Bamberger, Else, Bamberger, Beil, & Schulte, 1999). Most of these studies have examined the effects of acute progestogen treatment and the current study administered hormones for approximately seven months in order to evaluate the long-term effects of hormone treatment. It has been found that chronic treatment of ovarian hormones results in different outcomes than more acute treatments (Gibbs, 1997; Morissette & Di Paolo, 1993; Pazol, Northcutt, Patisaul, Wallen, & Wilson, 2009). The long-term hormone treatment used in the current study may result in receptors that are less sensitive and the two progestogens may result in differential receptor sensitivities after long-term exposure.

The mPFC in rodents plays a role in higher-order cognitive behaviors such as working memory and behavioral flexibility (Birrell & Brown, 2000; De Bruin et al., 2000; Kolb, 1990). Many of these behaviors are impaired during aging. For example, aged rats showed decreased performance, as compared to young rats, on object recognition (Wallace et al., 2007) and delayed spatial alternation tasks (Ando & Ohashi, 1991; Tanila, Taira, Piepponen, & Honkanen, 1994). There is evidence that performance on tasks mediated in part by the PFC is correlated with

dopamine levels in the PFC of aged female rats (Luine, Bowling, & Hearns, 1990) and tyrosine hydroxylase fibers in the male mPFC (Mizoguchi et al., 2009). Therefore, the greater amount of tyrosine hydroxylase found in the mPFC in the current study would be expected to result in improved performance on tasks mediated by the prefrontal cortex. Indeed, our lab has shown previously that long-term treatment with estradiol and MPA improved acquisition of the t-maze in aged female rats (Chisholm & Juraska, 2012). However, when a subset of these animals was tested on the water-maze, this same treatment impaired performance as compared to all other hormone treated groups (Lowry, Pardon, Yates, & Juraska, 2010). Interestingly, estradiol treatment affects monoamine levels differently in the PFC and the hippocampus. Estradiol increased levels of monoamines in the PFC but decreased them in the hippocampus of young adult animals (Inagaki et al., 2010). The water-maze is thought to be heavily mediated by the hippocampus, while several brain regions are important for t-maze performance including the PFC and striatum (Lalonde, 2002; Redish & Touretzky, 1998). Although the current study did not look at tyrosine hydroxylase densities in the hippocampus, it is possible that estradiol in combination with MPA does not alter dopamine in the hippocampus providing an explanation for the differential behavioral outcomes observed in our previous studies.

Tyrosine hydroxylase is the rate limiting enzyme in the biosynthesis of catecholamines and therefore identifies both dopaminergic and noradrenergic fibers. However, there is evidence that suggests the changes found in the current study are primarily dopaminergic. Studies using dopamine β hydroxylase (DBH), a marker for adrenergic and noradrenergic fibers, in the primate prefrontal cortex have found a low density of fibers that were large in diameter whereas tyrosine hydroxylase staining was dense and labeled slender highly varicose axons (Lewis, Foote, Goldstein, & Morrison, 1988; Lewis & Morrison, 1989). There also appears to be distinct

morphologies between DBH and tyrosine hydroxylase fibers in rodents. When noradrenergic fibers were selectively depleted in the anterior cingulate of the rat, the remaining fibers were characterized as thin delicate fibers with many varicosities and presumed to be dopaminergic (Berger, Tassin, Blanc, Moyne, & Thierry, 1974). The fibers that were observed in the current study were thin and highly varicose more closely resembling those previously described as dopaminergic. Importantly, ovariectomy has been shown to increase DBH labeled fibers in the prefrontal cortex and this was reversed after estrogen or estrogen with progesterone treatment (Kritzer & Kohama, 1999). Based on this, any staining of noradrenergic fibers in the current study would be expected to increase the number of fibers in our ovariectomized animals and therefore underestimate the effects of hormone treatment observed on tyrosine hydroxylase fibers.

Long-term hormone treatment did not alter neuron number in the mPFC. Thus the preservation of the number of mPFC neurons in intact aging females compared to males observed by Yates et al. (Yates et al., 2008) is not accounted for by the hormone replacement regiment given here. These results may seem surprising due to the numerous studies that have demonstrated that estrogens are neuroprotective in young animals and in vitro (Garcia-Segura et al., 2001). However, there is a growing body of research indicating that the adult brain and aged brain respond differently to hormone treatments. For example, estrogen treatment reduced cortical infarct volume in young adult animals, but resulted in greater infarct volume in middle-aged animals (Selvamani & Sohrabji, 2010b). While replicating this finding, a follow up study found that estrogen treatment increases levels of IGF-1 in young animals, while decreasing levels in middle-aged animals (Selvamani & Sohrabji, 2010a). Therefore it is important that studies examining the effects of hormone treatments on problems associated with aging do so in an

appropriate model. In addition, it is unknown how neuroprotective properties seen after extreme insult relate to normal aging.

This is the first study to find that long-term treatment with estradiol alone or in combination with MPA alters the prefrontal dopaminergic system during aging. Because the deterioration of the dopaminergic system during normal aging is correlated with decreased performance on cognitive tasks, the results provide a possible mechanism by which hormone treatments could benefit cognition during aging.

Figures and Tables

Figure 11.

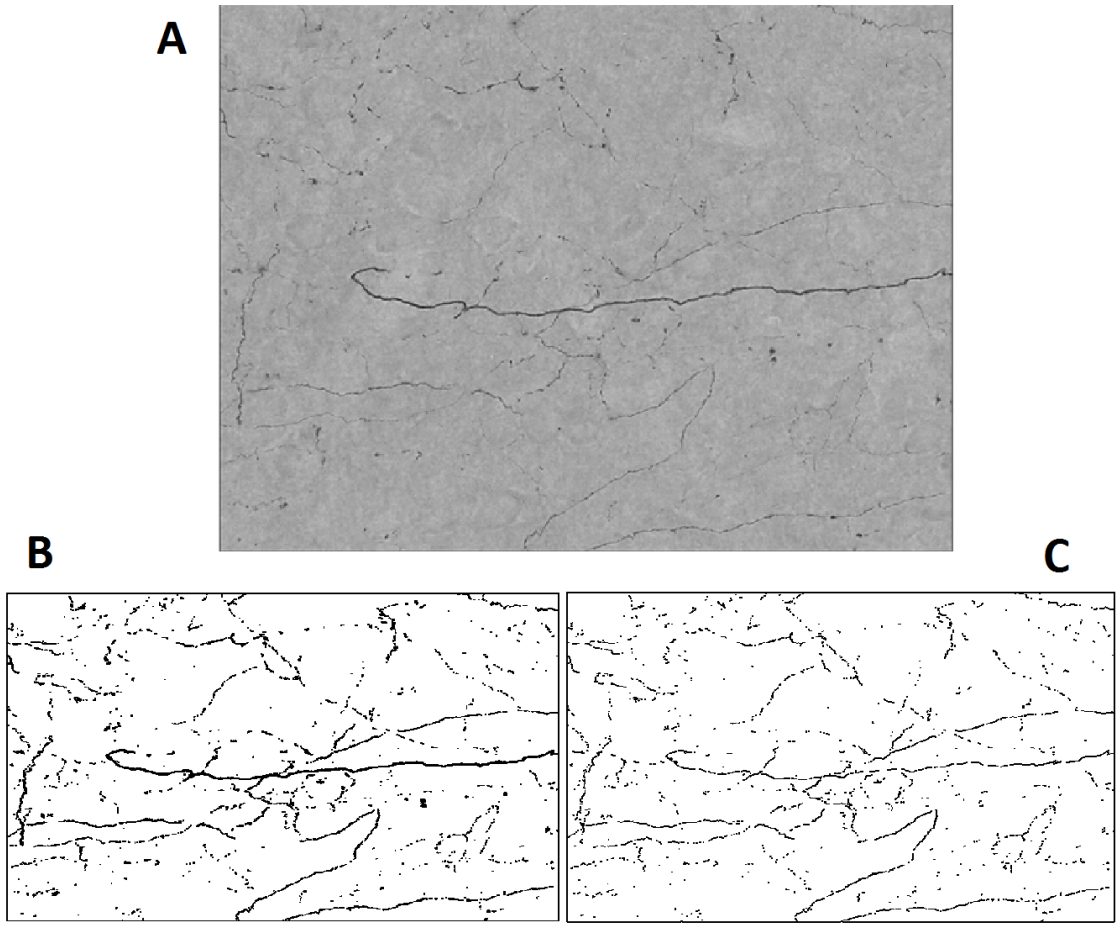

Figure 11. A. Tyrosine hydroxylase immunostained fibers in a Z-stacked image that has been compressed using Axiovision software. Image pixel density (the percent of the image in black) was measured in two ways: first as a binary image (B), taking thickness into account, and then as a skeletonized image (C), reducing the thickness to 1 pixel wide.

Figure 12.

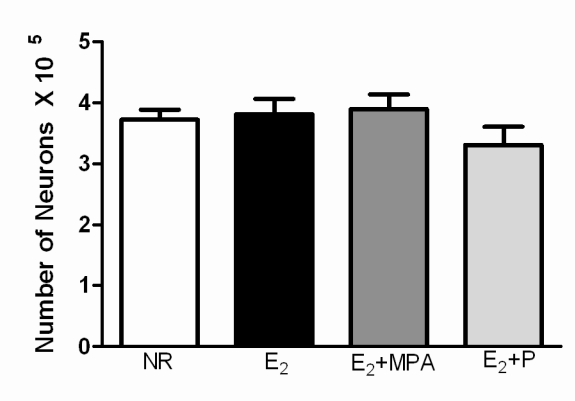

Figure 12. The total number of neurons in the mPFC (mean+ SEM). There was not a significant effect of hormone treatment on neuron number.

Figure 13.

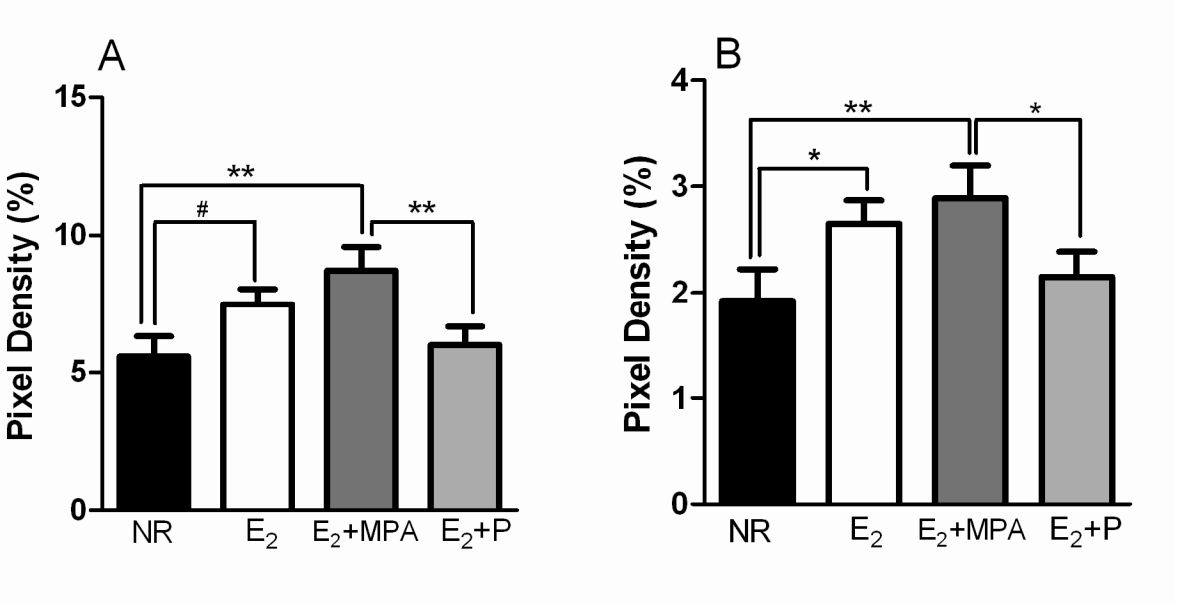

Figure 13. The density of TH fibers in Layer 1 (mean+ SEM). (A) In binary images, there was an effect of hormone treatment in ($p < .03$). (B) In skeletonized images, there was a trend for an effect of hormone treatment in ($p < .09$) * ($p < .05$), # ($p = .06$) $ ($p < .08$).

Figure 14.

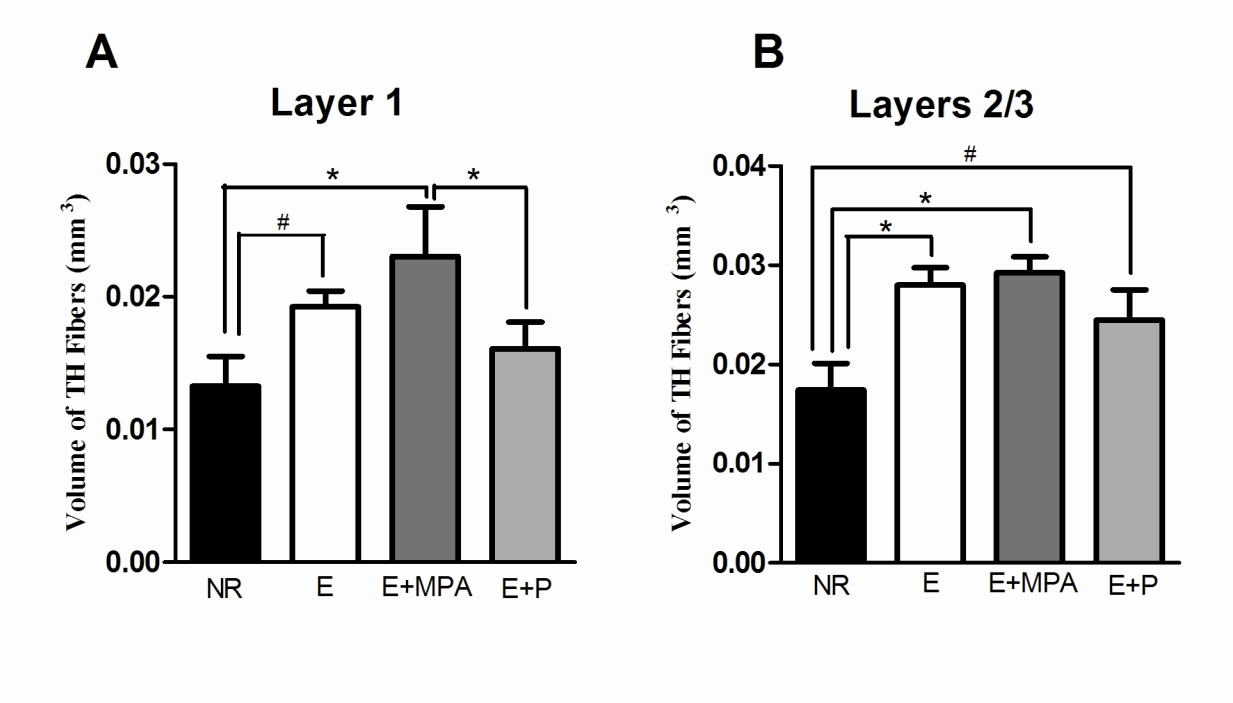

Figure 14. The volume of TH fibers (mean+ SEM) within the mPFC. In layer 1 (A) ($p<.03$) and layers 2/3 (B) ($p<.04$) there was an effect of hormone treatment. * ($p <.02$), # ($p < .07$).

Figure 15.

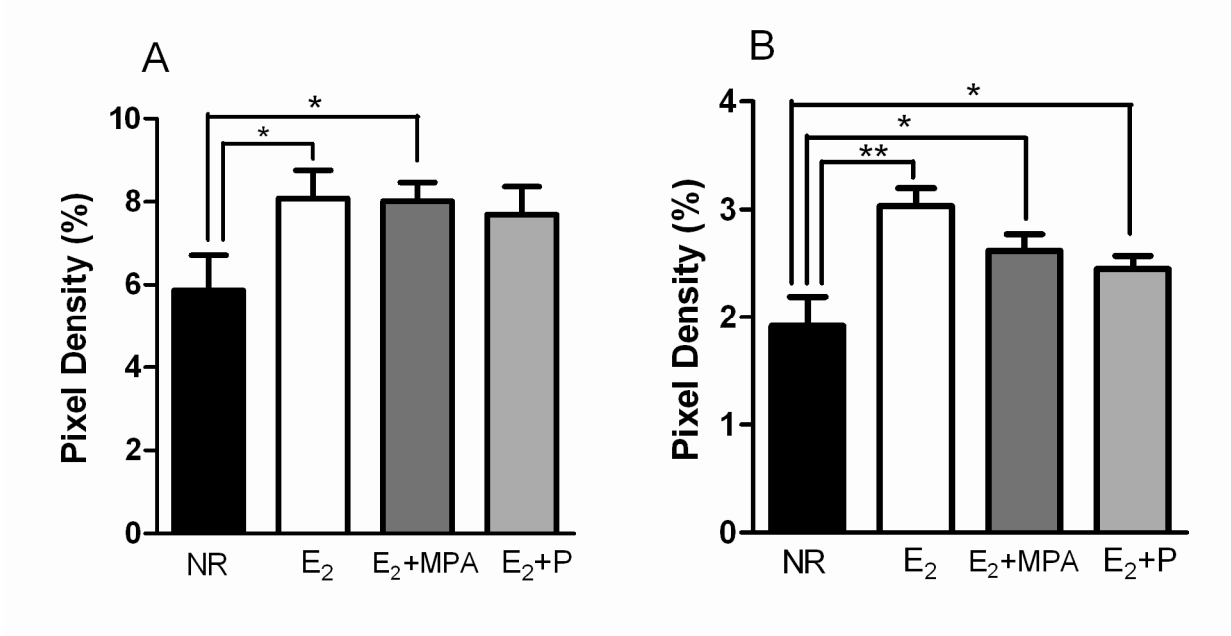

Figure 15. The density of TH fibers in layers 2/3 (mean+ SEM). In binary images (A) there was an effect of hormone treatment ($p < .05$). In skeletonized images (B), there was an effect of hormone treatment in Layers 2/3 ($p < .01$). * ($p < .05$), # ($p < .06$).

Figure 16.

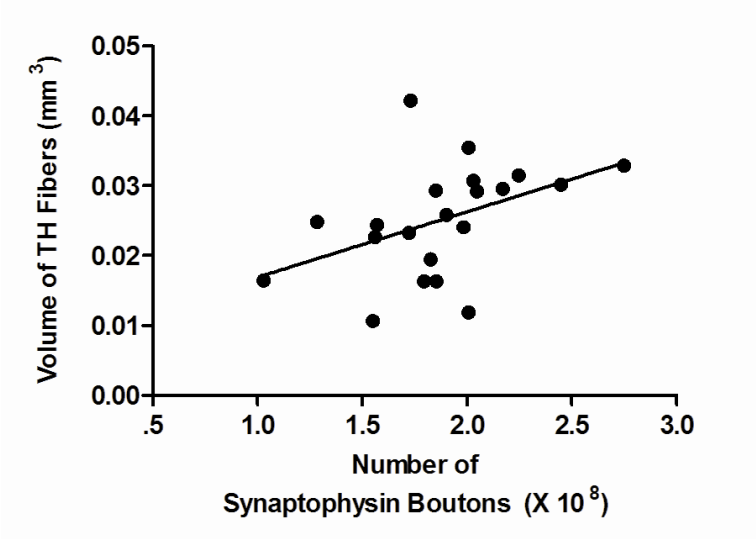

Figure 16. The correlation of the volume of tyrosine hydroxylase and synaptophysin labeled boutons in layers 2/3 of the medial prefrontal cortex ($r = .44$, $p < .05$)

Table 4. Mean Body and Uterine Weight for all subjects

Hormone Group	Mean Body Weight (g)	Mean Uterine Weight (g)
No replacement	585.2 ± 21.9	.09 ±.01
Estradiol	454.1 ± 27.6*	.16 ±.02*
Estradiol & P	458.4 ± 28.3*	.12 ± .01*
Estradiol & MPA	404.7 ± 28.3*	.16 ± .02*

Body and uterine weights were taken at sacrifice for all groups. No replacement animals weighed significantly more and had lower uterine weights that all hormone treated groups. * $p < .01$

CONCLUSIONS

After the negative results from the Women's Health Initiative many women and healthcare providers questioned the benefits of hormone replacement therapies. However since this time, it has become evident that the timing of hormone initiation is critical for the beneficial effects of hormone treatment, an idea now known as the window of opportunity

The series of experiments conducted for this dissertation found no adverse effects of any hormone combination started in middle age when compared to control animals. In fact, a beneficial effect of estrogen in combination with MPA was found for several measures including acquisition of a delayed alternation task and synapse number and tyrosine hydroxylase fiber density in the mPFC. Although this series of experiments did not directly examine the window of opportunity, it does add to the literature showing beneficial effects of hormone treatment when it is initiated soon after the loss of naturally circulating hormones. The studies presented here are the first to examine the long-term effects of estrogen in combination with MPA and the beneficial outcomes found in the current experiments highlight the need for future studies examining the cognitive and neural effects of this hormone treatment. Given the differences in how the hippocampus and mPFC age, it would be important to determine if estrogen in combination with MPA has similar effects on these two brain regions during aging.

In addition, whereas many studies in young animals find beneficial effects of estrogen only treatments, this series of experiments found that the addition of MPA led to a greater effect than estrogen only. As discussed in previous chapters, there are many changes that occur during aging and therefore it is not reasonable to expect similar effects in the young and aged brain. Because of this, future studies should use aging animals when attempting to model the effects of hormone treatments during menopause.

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